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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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STUDIES OF THE LYMPHATIC TISSUE.

III. Experimental Studies of the Relation of the Lymphatic Tissue to the Number of Lymphocytes in the Blood in Subcutaneous Infection with Staphylococci.

By WILHELM EHRICH, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Plates 25 and 26.

(Received for publication, October 16, 1928.)

In two papers published elsewhere the cellular structure of the secondary nodules (1) and their embryological development in human beings (2) have been described. It was found impossible to accept the conception formulated on Flemming's original theory that Flemming's secondary nodules are the places of formation of the lymphocytes of the blood, or to accept even a modification of this theory without some doubt.

If the conception of Hellman (3, 4) and his pupils is correct, that Flemming's secondary nodules represent "reaction areas" to "toxic" irritations of low intensity, it should be possible to induce a development of them experimentally by such irritations so that they could be studied in all stages of their development. The origin of the lymphatic hyperplasia which appears upon "toxic" irritation furthermore should be demonstrable. Finally, by simultaneous counts of the lymphocytes in the blood, a relation might be found between possible lymphocytosis and the morphological changes in the lymphatic organs.

Such experiments have so far not been reported in the literature (*cf.* also Whitney (5)). The secondary nodules have indeed not been studied experimentally at all save by experiments in which destruction of secondary nodules by x-ray (Heineke (6)), benzene (Selling (7)), or by arsenic (Wätjen 8, 9)) was followed.

Material and Method.

These experiments concern the relation of subcutaneous infection to changes of the lymphatic tissue, mainly in the regional lymph nodes, and a comparison of the latter with the number of lymphocytes in the blood. The experiments were made with rabbits. For producing the infection staphylococci of low virulence were chosen. They were injected under the skin of one hind foot, because in rabbits the regional lymph nodes are united in one popliteal lymph node, the lymph node of the other leg forming a suitable control. Especial emphasis was laid on as constant experimental conditions as possible. In order to exclude mistakes which might be caused by differences in development of the lymphatic tissue (Hellman (10)) and by the different numbers of leucocytes (Lindberg (11))

TABLE I.

The Leucocyte Counts in Relation to Rabbit Color.

Color	No. of rabbits	Total amount of leucocytes (maximum-minimum)	Polymorphonuclear leucocytes	Lymphocytes	Monocytes	Mast cells
Black and mainly black.....	13	10,894 (6,675–13,925)	36	60	3	1
Half black and half white.....	4	8,030 (6,950–9,225)	37	61	1.5	0.5
Gray and brown.....	5	6,880 (5,850–7,425)	40	58	1.5	0.5
Mainly white.....	3	5,183 (4,650–5,925)	40	57	2.5	0.5
Albinos.....	3	4,584 (2,900–5,700)	43	51	5.5	0.5

at different ages, only animals of nearly equal ages were used. If the ages were not known, animals of the same weight were taken, which Hellman found (10) may be done with young animals of the same race. Rabbits of 1,000 to 1,500 gm. were chosen since they were at the age in which lymphatic tissue is well developed and diseases are, for the most part, still absent. Some older animals were studied for comparison. Only animals as nearly alike as possible in color were used in each series, because it has been found that such animals with dark fur as were used had more leucocytes as well as more lymphatic tissue than lighter animals. All were males.

The leucocyte counts of 28 normal animals weighing about 1,000 gm. each, have been collected (Table I). The counts were repeated several times under as constant conditions as possible. The blood was always taken between 11 and 12 a.m., and from each animal at the same time each day in order to avoid the diurnal variation which has been described by Sabin, Cunningham, Doan and Kindwall

(12) and by Tschishikow (13). As the animals had not been fed since early morning digestive influences on the blood count are improbable. The absence of pathological lesions was determined by autopsy. The difference between the counts of the light and the dark animals refers especially to the lymphocytes. There is also to be noted a difference in the weight of the lymphatic organs in 13 animals (Table II). Especially marked is the difference in the weights of the peripheral lymph nodes and of the thymus. The weight of the mesenteric lymph nodes seems to be rather less in dark animals than in light ones. It is difficult, however, to decide how much is due to the lymphatic tissue because the ratio between the cells and the sinus portion, the quantity of which is determined by the

TABLE II.

*Relative Weights of Lymphatic Organs and Thymus in Light and Dark Animals.**

	Color	No. of rabbits	Weight	Axillary, cervical and popliteal lymph nodes together	Pancreas Aselli	Spleen	Thymus
			gm.				
I	White and gray	3	600	0.44	1.80	0.54	0.73
II		1	750	0.67	1.67	0.33	0.93
III		1	970	0.31	2.37	0.52	0.72
IV		1	1,175	0.30	1.40	0.39	0.70
I	Black and mainly black	1	590	0.43	0.60	0.25	1.19
II		2	760	0.64	1.05	0.36	1.41
III		1	980	0.61	2.04	0.41	1.12
IV		3	1,210	0.56	1.56	0.34	1.25

* The weights of the organs have been divided by the kilo weights of the rabbits

regional relation to the intestine, is extremely variable. The weight of the pancreas Aselli of the rabbits is also difficult to measure accurately because it is imbedded in a very varying quantity of fat, which cannot be excluded in weighing, because it cannot easily be separated from the lymph nodes. The irregularity in the weight of the spleen is probably not significant being due in the main to the varying content of the blood.

For all series the same pipettes and counting chambers were used. Two to three counts were made always and in the differential 400 to 600 leucocytes were counted in two smears.*

* In the differentiation of lymphocytes from monocytes and histiocytes in blood smears, all mononuclear cells which were not recognized with certainty as monocytes or histiocytes, were counted as lymphocytes.

Beside the popliteal lymph nodes the axillary, retroperitoneal, mesenteric lymph nodes, the lymph nodes of the intestine and the spleen were studied. The organs were fixed, cut and stained, as described in a previous paper (1).

Protocols.

Series 1 consisted of six rabbits of one brood, about 6 months old, of brown-black color. They were injected subcutaneously with 1 cc. of a 48 hour broth culture of *Staphylococcus aureus* of especially low virulence (obtained from the surgical clinic in Freiburg) diluted with equal parts of saline solution. At 2, 4, 6, 9, 12, 18, 21 and 29 days after injection leucocyte counts were made on all animals still living. R 15 was killed after 2 days, R 14 after 4, R 16 after 6, R 12 after 14, R 13 after 21 and R 11 after 29 days.

Series 2 consisted of eight rabbits of about 1,300 to 1,400 gm. They were injected subcutaneously with 1 cc. of a 48 hour broth culture of *Staphylococcus aureus* diluted with saline solution in equal parts. The leucocytes of these animals were counted beforehand, and after the injection only when the animals were to be killed. R 78 was killed after 1 day, R 76 after 2, R 72 after 3, R 79 after 5, R 71 after 7, R 73 after 14, R 70 after 28 and R 76 after 42 days. R 72 showed septicemia (streptococci) at autopsy and R 70 bronchopneumonia (*Bacillus lepisepticus*) and were consequently excluded from further studies.

Series 3 consisted of five rabbits about 2 years old and of dark color. Infection and the counting of leucocytes were managed as in series 2. R 6B was killed after 3 days, R 7B after 5, R 3B after 7, R 11B after 14, R 10B after 35 days. R 6B showed bronchopneumonia of both sides and pleurisy at autopsy, and R 7B fresh and R 3B older pneumonic areas in the lungs. R 6B and 7B were excluded from further study, whereas 3B was used for comparison.

Series 4 consisted of five rabbits about 1,200 gm. in weight, of black-brown color. Staphylococci were injected as in series 2 and 3, but the leucocytes were not counted, because this series was used only for studying the histological changes during the first days after injection. R 159 was killed after 1 day, R 160 after 1½, R 161 after 2, R 162 after 2½ and R 163 after 3 days.

RESULTS.

The results of the experiments will be considered together because those of the four series agree closely. At the site of injection a hemorrhagic inflammation first developed. On the 7th day in all series small abscesses were found at the same site, the contents of which in series 1 and 2 thickened after 14 days and consisted after 35 days of only a small amount of pus. In the old animals of series 3, however, the abscess increased in size from the 7th day onward and after 42 days (R 77) embraced a large part of the leg.

The regional popliteal lymph nodes in all cases were swollen markedly from the 1st day onward and were often about 5 times the size of the lymph node of the other leg. The color of the regional node of the infected leg was normal.

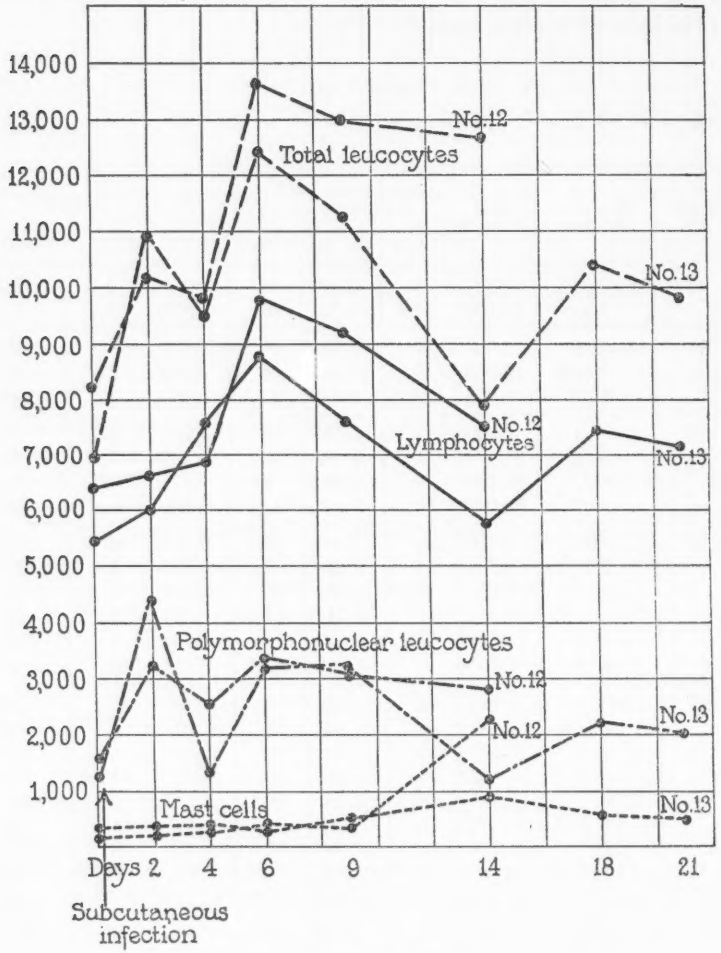
TABLE III.

Changes in the Leucocyte Count after Subcutaneous Infection with Staphylococci (Series 1).

Rabbit No.	Time after injection	Total leucocytes	Polymorphonuclear leucocytes		Lymphocytes		Mast cells	
			Per cent	Absolute	Per cent	Absolute	Per cent	Absolute
15	Control	9,250	28	2,590	69	6,382	2.5	231
	2 days	10,800	34.5	3,726	62	6,696	2	216
14	Control	9,700	31	3,007	66	6,622	2	194
	2 days	10,000	34	3,400	63	6,300	1	100
	4 "	9,000	27.5	2,475	71	6,390	1	90
16	Control	8,800	17.5	1,540	80	7,040	2.5	220
	2 days	10,450	34	3,553	63	6,583	1	104
	4 "	11,700	20	2,340	78	9,126	1	117
	6 "	11,000	24	2,640	72	7,920	2	220
12	Control	8,250	19	1,567	78	6,435	2	165
	2 days	10,200	32	3,264	65	6,630	2	204
	4 "	9,850	26	2,561	70	6,895	3	295
	6 "	13,650	25	3,412	71.5	9,760	3	409
	9 "	13,000	24	3,120	71	9,230	2.5	325
	14 "	12,700	22	2,794	59	7,493	18	2,286
13	Control	6,950	18	1,251	78.5	5,456	3	208
	2 days	10,950	40	4,380	55	6,022	3.5	383
	4 "	9,500	14	1,330	80	7,600	4	380
	6 "	12,450	26	3,237	70.5	8,777	3	373
	9 "	11,250	28	3,150	68	7,650	4	450
	14 "	7,900	15	1,185	72	5,688	10	790
	18 "	10,400	21.5	2,236	72	7,488	5	520
	21 "	9,800	21	2,058	73	7,154	5	490
11	Control	10,950	14	1,533	82	8,979	3	328
	3 days	11,800	30	3,540	67	7,906	2	354
	29 "	11,000	16	1,760	81	8,910	2	220

Leucocyte Counts.—In Table III are given the leucocyte counts of all rabbits of series 1. In Text-fig. 1 curves are drawn of the counts

of R 12 and 13 of this series. The highest point of polymorphonuclear leucocytosis is probably not expressed in Text-fig. 1 (series 1) be-



TEXT-FIG. 1.

cause it apparently occurs before the 2nd day, as was shown by series 2 and by a further experiment. The number of lymphocytes at first

decreased and had already reached the lowest point before the 2nd day. They then increased slowly and reached the highest point in series 1 at the 6th to 9th day and in series 2 at the 7th day. They then decreased again and, as with the other leucocytes, were found normal in number in all instances after the 3rd week. There was a mast cell leucocytosis at the 14th day in series 1 and 2.

While series 1 and 2 behaved in the same way the number of leucocytes in the old animals showed marked differences. Whereas the normal proportion of polymorphonuclear leucocytes to lymphocytes on the average was 25:75 in the first two series, it was 51:49 in the old animals (series 3). The number of lymphocytes in them always remained below the number of polymorphonuclear leucocytes. The highest point of the polymorphonuclear leucocytosis and of the lymphocytosis occurred later than in the young animals and the mast cell leucocytosis was apparently wanting.

Histology of the Lymph Nodes.—The histological changes in the regional lymph nodes agreed very closely in the different series. During the first 2 or 3 days a purulent hemorrhagic lymphadenitis was the outstanding pathological change. It is well known (*cf.* Kankaanpää (14)), so it need not be described again. The changes in the lymphatic tissue and especially in the secondary nodules which predominated from the 3rd day onward merit detailed description.

The secondary nodules which are normally present (Fig. 1), showed regressive changes in all series during the first few days. Polymorphonuclear leucocytes and hemorrhages often occurred in them. The nuclear fragments increased in number. The boundaries of the secondary nodules were lost. In a few nodules reticular centers appeared. In a few animals a number of Flemming's secondary nodules also appeared, but were regressively changed later. At the same time, at the margin of the cortex new small solid secondary nodules were formed.

In this period (the first 2 to 3 days) the proportion between medulla and cortex changed in favor of the medulla, owing chiefly to the accumulation of fluid in the sinus but partly to purulent hemorrhagic lymphadenitis. But in the days following an enormous enlargement of the cortex took place starting in series 1 on the 4th day (R 14 and R 16), and in series 2 and 4 on the 2nd day (R 76, R 79, R 162 and R 163). Shortly before the highest point of the lymphocytosis was reached the cortex occupied so large a part of the lymph nodes that in most instances only few central sinuses could be found (Fig. 3). The cortex consisted especially of small lymphocytes and contained a number of well developed veins into which

numerous lymphocytes migrated (Fig. 6). In the margin of the cortex smaller and larger solid secondary nodules were found.

The transition between the two pictures just described is shown in Figs. 4 and 5. Fig. 4 (R 78) shows an enlarged lymph node with an hemorrhagic purulent lymphadenitis. The cortex contains larger and smaller solid secondary nodules, few transition forms and also two small pseudo-secondary nodules.

Fig. 5 shows a further phase. On one side, some transition forms are still found, the centers of which contain many polymorphonuclear leucocytes. On the other side, some solid secondary nodes are seen, which are smaller than the transition forms. The largest part of the cortex, consists, however, of one enormous pseudo-secondary nodule which invades the tissue in all directions. On its external margin single small solid secondary nodules are found.

This pseudo-secondary nodule consists especially of small lymphocytes and contains distinct veins and reticular fibres. Everywhere large cells with karyokinetic figures are seen often connected with the walls of the vessels (Fig. 7) and penetrating the tissue. These cells have large round nuclei with fine networks of chromatin and large nucleoli. The protoplasm is broad and intensely basophil. Stained by the method of Schridde these cells contain very small or moderately sized, more or less, round mitochondria in the arrangement described by Schridde (15, 16). These cells are described by Marchand (17) as proliferating endothelial (reticular) cells. They differ from the so called lymphoblasts by their finer chromatin structure, by the very insignificant thickening of the nuclear wall and by their much broader and much more basophilic protoplasm. From these cells all transitions to small lymphocytes as well as to reticular cells are found. So called lymphoblasts, such as are found in Flemming's secondary nodules, are however, missing entirely. No myeloid cells are to be seen.

In the hyperplasia of the lymphatic tissue not only does the cortex take part, but there is also in the medullary cords an intense proliferation of lymphocytes (Fig. 5). There are many karyokinetic figures. Solid secondary nodules appear later.

Only when lymphocytosis reached its highest point in the blood stream did Flemming's secondary nodules appear. In series 2 they began to appear after 7 days (starting with R 71) but in series 1 only after 14 days (from R 12 on), for in this series no lymph nodes were available for study between the 7th and 9th days. The first of Flemming's secondary nodules to be seen lie partly in the margin of the cortex and mostly have large marginal zones, but some lie also in the inside of the cortex and in the medullary cords, where marginal zones are sometimes lacking. The light centers are at first of varying sizes but are generally small. They are typically built and often have central capillaries or arterial precapillaries.

When the number of lymphocytes in the blood began to fall, Flemming's secondary nodules grew in number and size and only when the number of lymphocytes again reached normal, did they reach their greatest development in number and size. This was reached in series 1 after 29 days (R 11) and in series 2 apparently after 28 days (R 70). The lymph nodes now seemed to consist nearly exclusively of large Flemming's secondary nodules with very large light centers rich in karyokinetic figures and distinctly limited against the narrow marginal zone. The latter was distinctly separated by reticulum from lymphoid tissue. This picture seemed to remain constant over a longer period. Our longest surviving animal (R 77) still showed this picture 42 days after the injection of the bacteria, that is to say for about 3 to 4 weeks after the ending of the lymphocytosis (Fig. 2).

In the old animals of series 3 conditions are very similar to those in the young animals. The first of Flemming's secondary nodules were found after 7 days (R 3B), and in enormous quantity and size after 15 days (R 11B) and still more so after 35 days (R 10B).

While the next more centrally lying regional retroperitoneal lymph nodes show these changes in part, the other lymphatic organs exhibit normal pictures. At the time of the most marked development of Flemming's secondary nodules in the regional popliteal lymph nodes, the secondary nodules in the other peripheral lymph nodes and in the spleen also seemed to be somewhat enlarged, but this appearance was found in its typical development only in rabbits 11B and 10B, which had both shown very large abscesses. In these animals very large Flemming's secondary nodules were found in all the peripheral lymph nodes which were studied, as well as in the spleen.

DISCUSSION.

From these experiments it can be seen that subcutaneous infection with staphylococci of low virulence results in lymphatic hyperplasia and the production of Flemming's secondary nodules in the regional lymph node. From the inflammatory irritation there first appears lymphadenitis and regressive changes of the existing Flemming's secondary nodules and transition forms which are then dissolved into lymphoid tissue. New solid secondary nodules appear at the margin of the cortex which grow, and as is shown so clearly by Figs. 1, 4, 5 and

3, lead to pseudo-secondary nodules and finally to diffuse lymphatic hyperplasia. That the pseudo-secondary nodules originate in Flemming's secondary nodules is improbable because in this case (Fig. 5) all existing secondary nodules with light centers show intense regressive changes with polymorphonuclear leucocytes and hemorrhages.

The more the lymphatic hyperplasia increases the greater is the increase in the number of lymphocytes. At the time of the greatest lymphocytosis (or shortly before) the lymph nodes still consist only of especially small lymphocytes while so called germinal centers or lymphoblasts are missing. Only when the highest point has been passed (or at the highest point) the first of Flemming's secondary nodules appear. They grow in number and size and reach their greatest development only when the lymphocytes in the blood have again decreased to normal. Here they remain quite a while. In the cases studied this remained the situation even in the last animal of the series which was killed, after the 42nd day.

In the preceding morphological and embryological studies of the secondary nodules it has been shown that Flemming's theory in its original conception cannot be accepted. These experiments show that lymphocytosis at least after subcutaneous infection with staphylococci has no relation to changes in the number and size of Flemming's secondary nodules, at least not in the regional lymph node. For at the time when the number of lymphocytes in the blood is highest, so called germinal centers are entirely wanting. The converse is also true; at the time of the highest development of Flemming's secondary nodules no increase in the number of lymphocytes in the blood can be found. The possibility that other lymphatic organs acted in a compensatory manner cannot be entirely excluded. There was at least no increase in number and no further development of Flemming's secondary nodules elsewhere to be found except those changes which were parallel with their development in the regional lymph nodes.

Against a modified Flemming's theory new doubt must be raised. If, according to the modified view, Flemming's secondary nodules are thought of as reserve depots of young lymphocytes it can be inferred from these experiments that whatever increase in formation of the nodules took place amounted to no more than an excess incident to the process of regeneration. But even this possibility seems to disappear

because Flemming's secondary nodules were changed regressively before the beginning of lymphocytosis, and the growth of the pseudo-secondary nodules took place without so called lymphoblasts, as they are found in Flemming's secondary nodules.

While no relation between lymphocytosis and Flemming's secondary nodules could be found, these showed on the other hand, a certain relation to the development of the abscesses. In each series Flemming's secondary nodules appeared only when abscesses were formed. They increased with the thickening of the abscesses in number and size. The greatest development in Flemming's nodules was found in the two animals with the largest abscesses (R 10B and R 11B).

There remains for discussion the question of how the lymphocytosis here comes about anatomically and which cells are responsible for the formation of small lymphocytes. The fact that the development of the pseudo-secondary nodules which leads to diffuse lymphatic hyperplasia is parallel with the increase of lymphocytes in the blood, makes it likely that pseudo-secondary nodules are responsible in this situation as in embryonic life for the formation of lymphocytes. This conception is supported by the observation that just at the time of greatest lymphocytosis enormous quantities of lymphocytes can be seen to migrate into the veins of the hyperplastic lymphatic tissue. The pictures which are familiar in lymphatic leucemia bear evidence of the correctness of this view. For in this disease Flemming's secondary nodules are almost entirely missing and the anatomical picture is one of diffuse lymphoid hyperplasia.

Is there a basis in these studies for drawing an inference as to the mother cells of the lymphocytes? In this connection it is necessary to speak only of some observations that were made in connection with growing pseudo-secondary nodules. Large cells were seen coming from the vascular walls. That these are rapidly multiplying is shown by their great number and the number of karyokinetic figures. These cells have been identified as being the proliferating endothelial (reticular) cells of Marchand (17). We find all transitions between these cells and the small lymphocytes as they are missing in Flemming's secondary nodules. We also find transitions to differentiated reticular cells. So called lymphoblasts, however, are missing here. It appears therefore to be reasonable to sup-

pose that the small lymphocytes are derived from Marchand's proliferating endothelial (reticular) cells.

To judge from transitional pictures genetic relations apparently exist also between lymphocytes on the one hand and histiocytes and monocytes on the other. Masugi (18) has recognized as the common mother cells of the latter the resting mesenchymal cells which are in no special stage of irritation and which exist as resting histiocytes of the connective tissue, as resting pulp cells, as resting reticular cells or as resting reticulo-endothelial cells. Pictures like Fig. 8 which shows a lymph node with a very narrow cortex and medullary cords, also point in this direction. Secondary nodules and pseudo-secondary nodules are entirely missing. And, nevertheless, an intense production of lymphocytes takes place. With high power (Fig. 9) the sinuses are seen to contain many small lymphocytes and cells which are in the stage of division and which are very similar to the cells here in question. All transitions between them are observed. It appears to be entirely reasonable to derive the small lymphocytes in these lymph nodes from resting reticulo-endothelial cells.

SUMMARY.

1. On subcutaneous infection of rabbits with staphylococci of low virulence there appears at the place of injection first a hemorrhagic-purulent inflammation and later a localized purulence. In the regional lymph nodes there is lymphatic hyperplasia, and in the blood a lymphocytosis.

2. In the regional lymph nodes there is first a regressive change of Flemming's secondary nodules and of transition forms. Then follows lymphatic hyperplasia, starting apparently from solid secondary nodules and progressing by way of pseudo-secondary nodules to a diffuse lymphoid hyperplasia. The increase of lymphocytes in the blood parallels this development.

3. Only after the highest point of the lymphocytosis has been reached or passed do we find the first Flemming's secondary nodules, which thereafter increase in number and size while the number of lymphocytes in the blood falls, and reach their maximum development when the number of lymphocytes in the blood is again normal. Therefore, the original conception of Flemming that the site of forma-

tion of the lymphocytes of the blood is in Flemming's secondary nodules, cannot be accepted.

4. The lymphocytes of the blood originate in the pseudo-secondary nodules as in embryonic life. The mother cells of the lymphocytes would appear to be Marchand's proliferating endothelial (reticular) cells.

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EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. Popliteal lymph node (rabbit). Normal popliteal lymph node with a number of solid secondary nodules (*a*) and transition forms (*b*). Longitudinal section. Iron-hematoxylin-eosin. ×15.

FIG. 2. Half popliteal lymph node. (R 77). 42 days after subcutaneous infection. The lymphatic tissue consists almost entirely of large Flemming's secondary nodules with only very narrow marginal zones. Longitudinal section. Iron-hematoxylin-eosin. ×15.

FIG. 3. Popliteal lymph node (R 76). 2 days after subcutaneous infection. Lymphatic hyperplasia. On the margin of the cortex a number of solid secondary nodules (*a*). Cross-section. Iron-hematoxylin-eosin. × 15.

FIG. 4. Popliteal lymph node (R 78). 1 day after subcutaneous infection. The cortex contains smaller and larger solid secondary nodules (*a*), few transition forms (*b*) and also two small pseudo-secondary nodules (*c*). Longitudinal section. Iron-hematoxylin-eosin. × 15.

FIG. 5. Popliteal lymph node (R 161). 2 days after subcutaneous infection. On the one side there are still some transition forms (*a*) with polymorphonuclear leucocytes in their centers. On the other side some solid secondary nodules (*b*). The largest part of the cortex consists of a pseudo-secondary nodule (*c*). Proliferating medullary cords (*d*). Longitudinal section. Iron-hematoxylin-eosin. × 15.

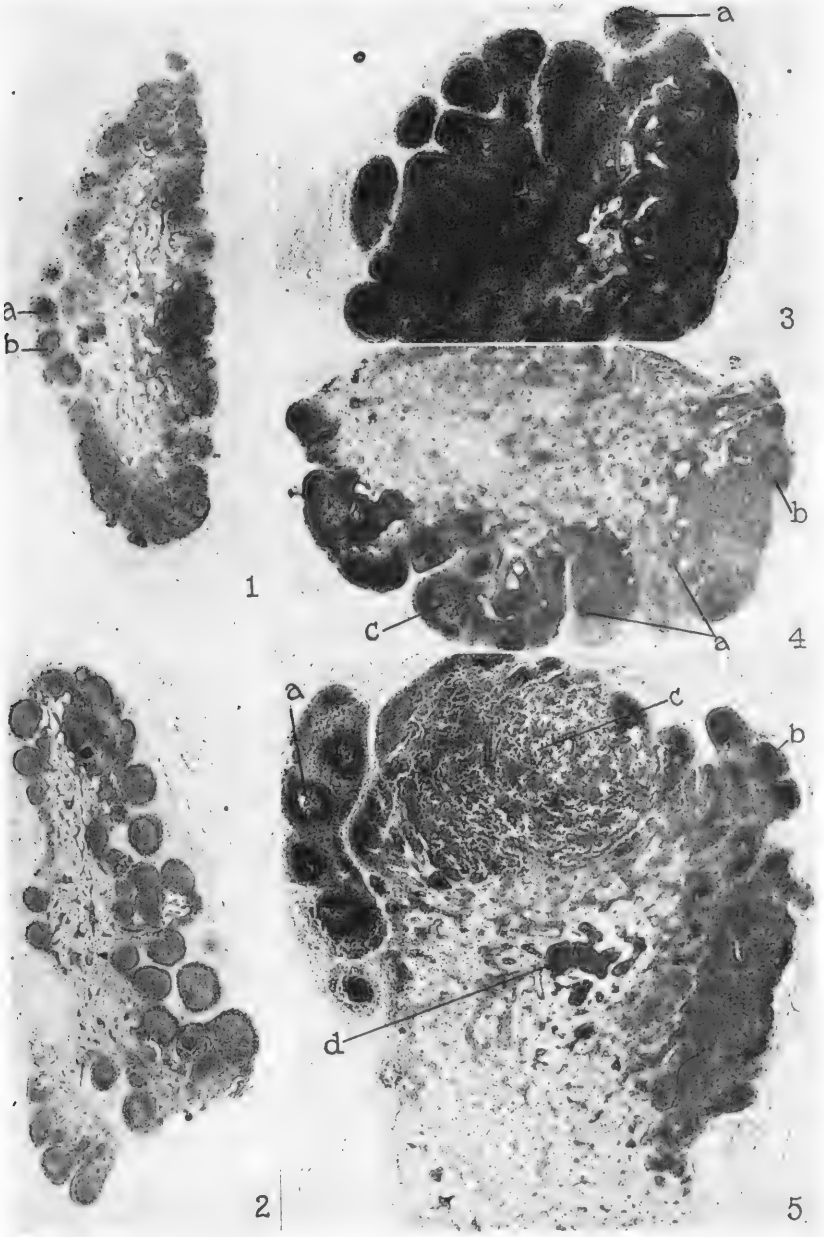
PLATE 26.

FIG. 6. Part of the cortex of Fig. 3 × 600. Many lymphocytes can be seen migrating into a vein (*a*). From the wall of the vessel some large cells have arisen (*b*).

FIG. 7. Part of the pseudo-secondary nodule of Fig. 5 × 600. From the walls of the vessels large cells have arisen (*a*) often with karyokinetic figures (*b*). From these all transitions to small lymphocytes can be seen (*c*).

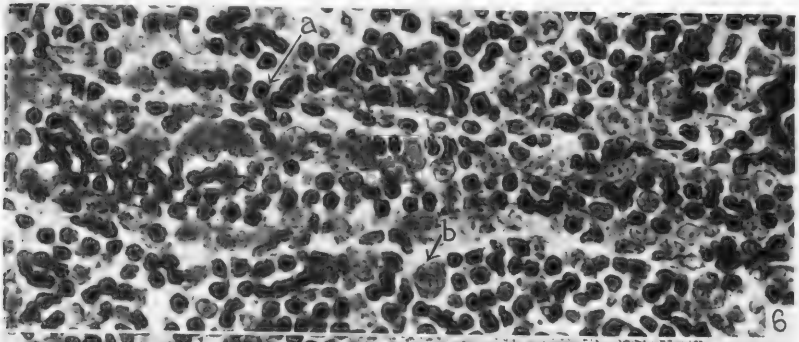
FIG. 8. Mesenteric lymph node (rabbit) with very narrow cortex and medullary cords. There are many lymphatic cells in the sinuses. Iron-hematoxylin-eosin. × 15.

FIG. 9. Part of Fig. 8 × 600. There are in the sinuses many small lymphocytes and large cells which are in the stage of division (*a*). Everywhere there is a large amount of pigment (*b*).



Photographed by Louis Schmidt.

(Ehrich: Lymphatic tissue. III.)



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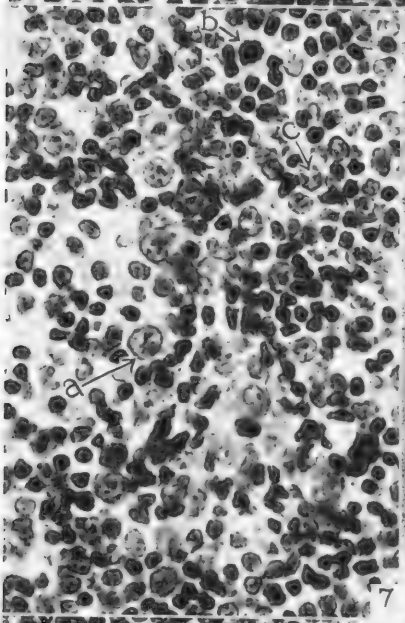

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Photographed by Louis Schmidt.

(Ehrich: Lymphatic tissue. III.)

STUDIES OF THE LYMPHATIC TISSUE.

IV. Experimental Studies of the Effect of the Intravenous Injection of Killed Staphylococci on the Behavior of Lymphatic Tissue, Thymus, and the Vascular Connective Tissue.

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Plates 27 to 30.

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It was shown in a previous paper (1) that after infection of the subcutaneous tissue with staphylococci of low virulence, diffuse lymphoid hyperplasia appeared in the regional lymph node, and that only later did Flemming's secondary nodules develop. At the same time lymphocytosis appeared in the blood, simultaneously with the appearance of lymphoid hyperplasia by way of pseudo-secondary nodules. Lymphocytosis had already disappeared before the greatest development of Flemming's secondary nodules in the regional node. The conclusion therefore was drawn that the lymphocytosis of the blood had its origin in the pseudo-secondary nodules and had nothing to do with Flemming's secondary nodules which made their appearance later.

In this study is reported the behavior of lymphatic tissue and of the lymphocytes in the blood after intravenous injection of killed staphylococci. A question at issue was whether by this means a general status lymphaticus or even a status thymicolymphaticus might be the response, or whether the spleen would play the main rôle in the reaction. The relation between the occurrence of lymphocytosis and changes in the lymphatic tissue were studied at the same time. Another question was, whether by the mere introduction of killed bacteria without reinjection, so called mesenchymal reactions would appear as a part of the process of immunization. Such reactions have been described by Oeller (2–5), Siegmund (6–8), Domagk (9–11),

Jacob (12) and Töppich (13); the views of these authors have received confirmation in the present study, at least in part. On the other hand, the existence of such reactions has been denied at least in part by Seemann (14–16), Gerlach and his associates (17–19) and partly also by Maximow (20) in conformity with general opinion.

Material and Method.

The material used consisted of 87 treated and 27 normal male rabbits. For reasons already given (1), animals as nearly alike in color and weight as possible were chosen.

The animals were treated partly with small and partly with large doses (in a manner determined by previous experiments during studies of immunization). The animals of series A were treated intravenously with varying doses of 20 hour whole broth cultures of *Staphylococcus aureus* killed by heating for 2 hours at 60°C. These animals will be referred to as treated with small doses. The animals of series B received intravenously killed staphylococci, which had been centrifuged and then diluted with saline solution, so that 1 cc. of the suspension corresponded to about 40 cc. of broth culture. These animals will be referred to as treated with single large doses.

In certain of the animals the leucocytes were counted at specified intervals according to the method already described (1). Curves showing their course will be discussed later. The animals were killed by a blow on the neck. The popliteal, axillary and cervical lymph nodes, the pancreas Aselli, thymus, spleen, liver, adrenals, heart, lungs and kidneys were weighed. All these organs were fixed, cut and stained in the manner previously reported (21).

Protocols.

Series A 1 consists of 17 rabbits of about 1,000 to 1,100 gm. The fur of half of them was light in color and of the other half dark. They received intravenously 6 daily small doses of 1 cc.; after a rest of 10 days they had a second similar series for another 5 days; and after a second rest of 9 days a third series of injections daily for 4 days. After 1, 2, 3, 4, 7, 10, 15, 17, 24, 28, 38 and 44 days the leucocytes were counted in 2 light and 2 dark animals; the animals were alternated so that no 2 were used on successive days. On the 1st day after the second and fourth injections (first series), on the 2nd and 10th days after the sixth injection, on the 5th day after the second series of injections and on the 5th and 11th days after the third series a light and a dark animal were killed (Text-fig. 3). Three animals died during the experiment from intercurrent diseases and were excluded from further study.

Series A 2 consists of 20 rabbits of about 800 to 1,500 gm., half of which were light and half dark. They were treated with small doses of 1 cc. during three

periods of 6 days each, with intermissions of 9 days between the periods. They were used mainly for microscopical study.

Series A 4 comprises 8 light colored animals weighing about 1,000 gm. They were treated for two periods of 6 days each with small doses of 5 cc. with an intermission of 9 days between the periods of immunization. The days on which the animals were killed are given in Table I.

Series B 1 consists of 38 rabbits weighing about 600 to 800 gm., 32 had light and 6 had dark fur. They received the sediment of 40 cc. of culture on four occasions at intervals of 1 week. In 4 light animals the leucocytes were counted after 6, 10, 14, 15, 17, 21, 24, 28 and 35 days. One of these animals as well as 3 of the other animals of this series died during the experiment and were excluded from further study. The days on which the animals were killed are given in Table I.

Series B 2 consists of 4 animals weighing about 1,000 gm., 2 of which were light and 2 dark. They were treated intravenously 4 times with 60 cc. of sedimented culture at intervals of 1 week and were killed 1 day after the first injection and others 5 days after the third and fourth injection.

RESULTS.

In reporting the results the changes in the lymphocyte count will be given first, then the weights of the organs and finally the anatomical changes in the organs.

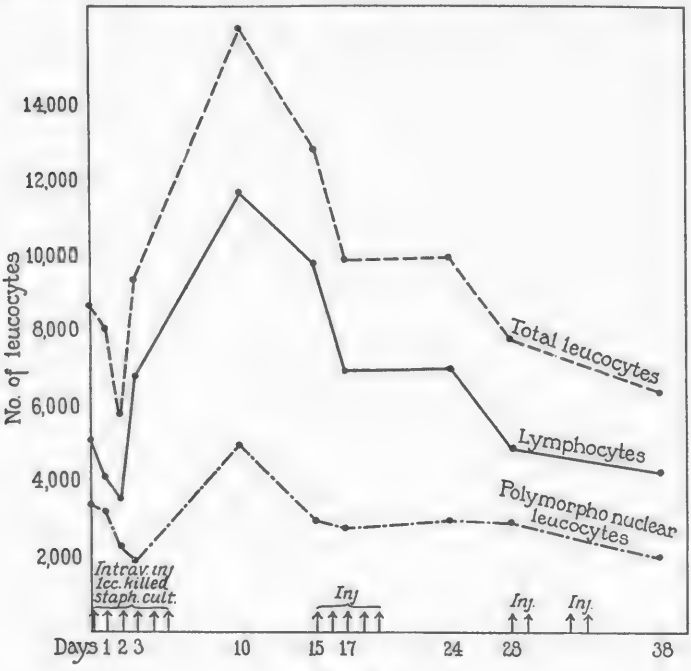
1. Lymphocytes.

The description of the curves of the lymphocytes can be given in a few words. Only changes in the lymphocyte and polymorphonuclear leucocyte counts are shown. In the differentiation of lymphocytes from monocytes and histiocytes in blood smears, all mononuclear cells which were not recognized with certainty as monocytes or histiocytes, were counted as lymphocytes.

In Text-fig. 1 the average numbers of leucocytes of 10 animals of series A 1 are given. The counts of 4 animals with slight coccidiosis at autopsy, were excluded from this curve; this disease, however, made very little difference either in the lymphocyte counts or in the weight curves; these results are in accord with the weights found by Hellman (22) in normal animals. The largest number of lymphocytes in the blood was found 10 to 14 days after the first injection in animals immunized with small doses. The number of lymphocytes increased about 125 per cent (Text-fig. 1).

Text-fig. 2 shows the numbers of leucocytes of a typical animal of

series B 1, treated with large doses. Two other animals showed similar curves, but at autopsy slight coccidiosis was present. Here also the greatest number of lymphocytes in the blood was found after about 14 days. The increase, however, amounted to only 70 per cent.



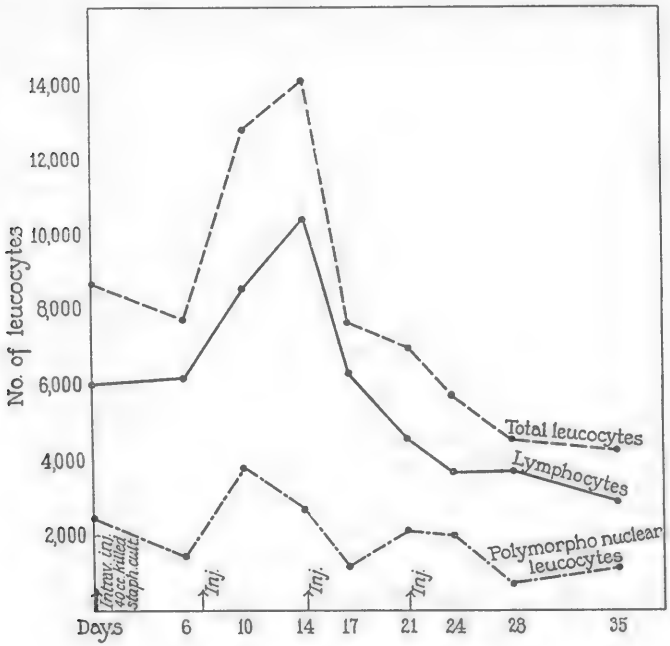
TEXT-FIG. 1.

2. Weights of the Organs.

In order to be able to compare the weights of the organs of each series the weights found at autopsy were divided by the kilo weight of the animals. The relative weights of the peripheral lymph nodes, spleen and thymus fluctuate in rabbits at these ages (Hellman (22)). In order to eliminate this source of error as well as those due to differences in length of life after the beginning of the injections, minor corrections according to Hellman's curves were made in the relative

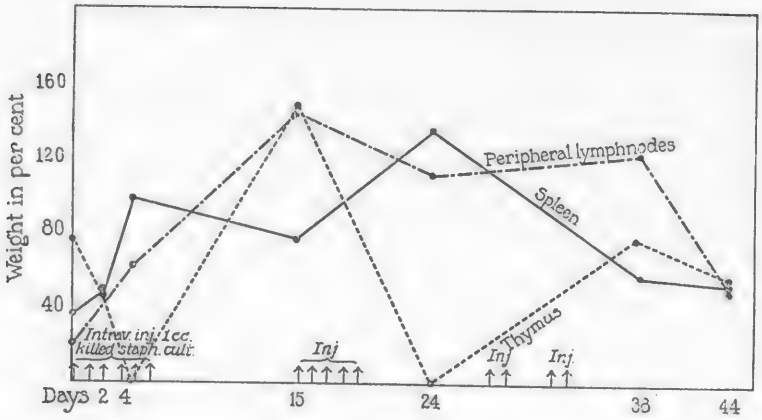
weights. The corrected weights have been expressed as a percentage of the normal weights, the averages of which are presented in curves.

(*a*) *Peripheral Lymph Nodes.*—The popliteal, axillary and cervical lymph nodes reacted similarly. They are therefore described together as peripheral lymph nodes. The changes in the weight of the lymph nodes of the 10 typical animals of series A 1 treated with small doses

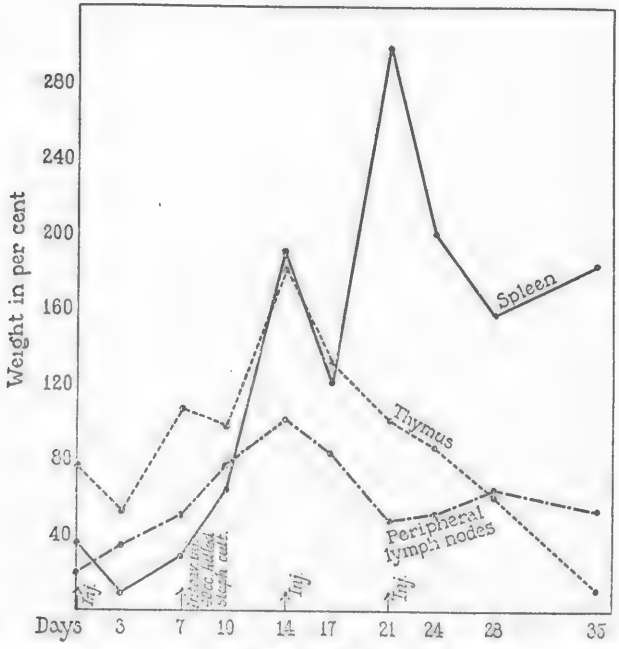


TEXT-FIG. 2.

are shown in Text-fig. 3. The weight of the lymph nodes increased slowly in these animals and reached the highest point after 14 days. Then it slowly decreased. The greatest increase in weight amounted to about 125 per cent and ran parallel to the increase in lymphocytosis. Animals of series A 4, which received 5 times the dose compared with series A 1, showed an increase of 120 per cent 24 days after the first injection.



TEXT-FIG. 3.



TEXT-FIG. 4.

In Text-fig. 4 the weight curve of the lymph nodes of 24 typical animals of series B 1 is given. The highest point was reached here, too, after about 14 days, but showed an increase of about 80 per cent only. The coincidence of the increase in weight of peripheral lymph nodes and of the number of lymphocytes in the blood is evident.

(*b*) *Pancreas Aselli*.—The weights of the mesenteric lymph nodes differed greatly, as might be expected from Hellman's findings. The varying amount of fat which cannot be separated from these lymph nodes apparently played a major rôle in these differences.

(*c*) *Spleen*.—In comparing the weights of spleens, the varying content of blood must be considered. It accounts for certain mistakes which could not be eliminated in Text-figs. 3 and 4. That it is possible to compare the weights of spleens is evident from Hellman's investigations as well as from our own curves, which could not be explained if the varying content of blood were a significant factor in the computations.

In the animals of series A 1, treated with small doses, the spleen weights increased slowly (Text-fig. 3), and reached their highest point, about 100 per cent, after approximately 4 weeks, that is to say, 14 days after the weights of the peripheral lymph nodes and the number of lymphocytes in the blood attained their maxima. The animals of series A 4 reacted similarly to those of series A 1. In the animals injected with large doses (series B 1), there was found, however, a much greater increase in weight (Text-fig. 4), which amounted to about 250 per cent after 3 weeks. The highest point occurred after the time when the peripheral lymph nodes and the lymphocytosis developed their maxima.

(*d*) *Thymus*.—In series A 1 a decrease in the weight of the thymus (Text-fig. 3) took place at first which, after the end of the injections of the 1st week, was followed after 14 days by an increase of 70 per cent. The next series of injections caused another decrease, whereas injections of the third series no longer influenced the curve. In the animals injected with large doses (Text-fig. 4) an enlargement of the thymus was also found after 14 days. The increase amounted to as much as 100 per cent. Later injections caused progressive atrophy.

(*e*) *Liver, Kidneys, Lungs, Heart and Adrenals*.—The weights of these organs were recorded regularly only in series B 1. No distinct

change was found except in the lungs which increased in weight. The adrenals varied a great deal.

3. *Microscopical Findings.*

(*a*) *Lymph Nodes.*—In the microscopical study of the lymph nodes the size, number and composition of the secondary nodules and pseudo-secondary nodules were mainly examined. In order to be able to compare them they were measured in all animals. The increase in weight of the lymph nodes was caused, on microscopical analysis, by a general increase of all parts of the lymphatic tissue, but more especially by an increase in size and partly in number of the pseudo-secondary nodules (Fig. 1). In some animals of series B 2 reticular and epithelioid centers were also seen in the secondary nodules of some lymph nodes.

(*b*) *Spleen.*—Whereas the microscopical changes were slight in the lymph nodes, they were considerable in the spleen, as was to be expected, because this organ, unlike the regional lymph nodes, serves the body as a whole.

A marked enlargement and increase in number of the Malpighian bodies and a change in their composition were observed. Although normal spleens usually show only transitional secondary nodules, there occurred in these immunized rabbits an enormous number of Flemming's secondary nodules (Fig. 2). In the animals treated with small doses (Table I, series A) Flemming's secondary nodules reached their greatest increase in number and size 2 to 4 days after the first, and 2 to 3 days after the second series of injections. They seemed to reach a further high point after the third series of injections, but unfortunately no animals were available in the period of 1 to 5 days after the last injection. In the animals treated with single large doses (Table I, series B) the increase of Flemming's secondary nodules, as a rule, was less and reached its greatest development about 3 days after the second and third injections.

Beside the changes in the secondary nodules, lesions were found which seem to be of a special kind and apparently belong to the so called mesenchymal reactions. Everywhere in the interstitial tissue as well as in the outer zones of the secondary nodules, but especially around the smaller vessels, there appeared intensely basophilic cells (Fig. 4), partly of the type of lymphocytes and plasma cells, and partly of the type of histiocytes and of reticulo-endothelial cells. The proliferations often reached considerable size. The cells showed many karyokinetic figures and often contained two or more nuclei. Often cells were found, which apparently were loosened from the walls of the vessels. Another feature of this reaction was the appearance of often numerous giant cells (Fig. 3) of the Langerhans type. When

developed they were frequently very large. Their nuclei were pale and poor in chromatin. When the cells were developed, their protoplasm was pale in the interior of the cell and often contained pigments, whereas at the periphery mostly a narrow basophilic margin was to be seen. Younger giant cells contained more basophilic protoplasm and showed nuclei also in their centers, so that they rather resembled foreign body giant cells. In addition to those found in the interstitial tissue, giant cells as well as basophilic round cells were also found in a small amount in the sinuses.

As is shown in Table I, mesenchymal reactions of this kind were observed rarely and in slight degree in apparently normal animals, whereas giant cells were entirely missing. In the animals of series A 1 and A 2 injected with small doses mesenchymal reactions were found as early as the 1st day after the fourth injection (first series) and showed their greatest development in the week after the second series of injections. Giant cells appeared on the 10th day after the first series of injections and were found with regularity only after the third series of injections without reaching large numbers. The series A 4 differed from series A 1 only by a greater development of giant cells. In the animals of series B (Table I) treated with single large doses the mesenchymal reactions were found from the 2nd day after the first injection and showed their greatest development from the 4th day after the second injection until the end of these series. Giant cells were first found 14 days after the first injection, and were seen with great regularity from the 1st day after the second injection, but attained their greatest development after the third injection.

Beside these changes myeloid areas were found in the spleens of 4 animals of series B.

(*c*) *Thymus.*—The histological study of the thymus was especially concerned with the question, whether the medulla or the cortex played the greater rôle in the change in weight. The myeloid cells which are found in the thymus of rabbits mainly under the capsule varied so much in normal animals that no influence of the injections could be recognized. Changes in Hassal's bodies were but slight.

The decrease in weight took place mainly in the cortical substance. The more the thymus decreased the more indistinct became the differentiation between medulla and cortex. Finally it vanished entirely (Fig. 5). As a rule the increase in the weight concerned both substances (*cf.* Figs. 6 and 7). In the animals treated with small doses the thymus gland decreased more and more and showed complete atrophy 4 days after the sixth injection. This was followed by regeneration and subsequent hyperplasia. The second series of injections caused further atrophy, whereas the third series exerted no

TABLE I.

*Reactions in Spleen, Liver and Lungs in Relation to the Time and Number of Intravenous Injections of Killed Staphylococci.**

No.	Weight	†	Time after last injection	No. of injections	Spleen				Liver		Lung	
					Secondary nodules		Mesenchymal reactions	Giant cells	Mesenchymal reactions	Giant cells	Mesenchymal reactions	Giant cells
					Flemming	Transition						
				Normal rabbits								
	gm.											
56	380	0			0	+++	0	0	0	0	0	0
54	590	0			0	+++	0	0	++	0	0	0
53	750	0			+	+++	0	0	+	0	0	0
55	780	0			0	+++	0	0	++	0	0	0
58	1200	0			0	+++	0	0	0	0	+	+
59	1240	0			0	+++	0	0	+	0	0	0
51	1730	0			0	+++	+	0	0	0	0	0
52	2130	0			0	+++	++	0	+	0	+	+
49	2460	0			0	+++	0	0	+	0	0	0
48	2740	+			0	+++	+	0	++	0	0	0
50	2800	0			+	++	0	0	+	0	0	0
				Series A 1 and A 2								
			hrs.									
124	2020	0	24	2	0	+++	0	0	+	0	−	−
129	1080	0	"	"	0	+++	0	0	+	0	−	−
130	1020	+	"	4	+	+++	+++	0	+++	0	−	−
131	1100	0	"	"	0	+++	+++	0	+++	+	−	−
40	1000	0	2	5	0	+++	+++	0	+	0	0	0
41	1140	+	24	"	+	++	+++	0	++	0	+	0
45	940	0	1½	6	++	++	+++	0	+	0	+	+
37	1680	0	2½	"	+++	+	++	0	−	−	−	−
38	1520	0	3½	"	0	+++	+++	0	−	−	−	−

			days									
126	1230	+	2	"	+++	+	++	0	−	−	−	−
132	1240	+	"	"	+++	+	+	0	++	0	−	−
42	1020	0	"	"	++	+	+++	0	+	0	0	0
177	1780	0	4	"	+++	++	++	0	0	0	−	−
180	1180	0	"	"	++	+++	+++	0	++	+	−	−
181	1200	+	9	"	+++	++	+	0	+	+	−	−
182	1320	0	"	"	0	+++	+++	0	+++	++	−	−
127	1240	+	10	"	++	++	+	+	++	++	−	−
142	940	0	"	"	++	+	+	+	++	++	−	−
178	1210	0	12	"	+	++	+++	0	++	++	−	−
179	850	+	"	"	+	++	++	0	++	++	−	−
			hrs.									
43	1000	+	2	2 series	0	+++	++	0	+++	0	++	+
46	1120	0	3	"	++	++	+++	0	+++	0	+	+
			days									
88	990	0	2	"	+++	++	+++	+	++	+	+	0
92	1200	0	"	"	+++	++	++	+	++	+	+	+
89	1050	0	3	"	++	+++	+++	0	+++	+	++	+
93	1030	0	"	"	+++	+	++	0	++	+	+	+
135	1150	0	5	"	+	+++	+++	+	++	+++	−	−
142	1040	0	"	"	++	++	+++	+	++	++	−	−
			hrs.									
36	2050	0	$2\frac{1}{2}$	3 "	++	+	+++	+	++	+	++	+
39	1740	0	$3\frac{1}{2}$	"	+++	++	+++	+	++	+	+	+
			days									
125	1360	0	5	"	+	+++	++	+	+	+	−	−
136	1560	0	"	"	++	+++	++	+	++	++	−	−
134	1440	0	11	"	++	++	0	+	+++	+	−	−
139	1400	0	"	"	++	+++	+	+	+	+	−	−

* The symbols 0, +, ++, +++, ++++ indicate the degree of the reactions, − has not been examined.
† Moderate coccidiosis, echinococcus or tuberculosis.

TABLE I—*Concluded.*

No.	Weight	†	Time after last injection	No. of injections	Spleen				Liver		Lung	
					Secondary nodules		Mesenchymal reactions	Giant cells	Mesenchymal reactions	Giant cells	Mesenchymal reactions	Giant cells
					Flemming	Transition						
				Series A 4								
	gm.		*days*									
85	980	0	1	3	0	+++	+	0	++	0	+	+
86	970	0	"	"	0	+++	++	0	++	+	0	0
81	1140	0	2	6	+++	+	+	0	+++	0	0	0
84	1120	+	"	"	+++	+	+	0	++	+	+	+
83	1240	+	9	"	++	+++	+	+	++	++	+	+
87	1230	0	"	"	+	+++	++	++	+++	+++	++	+
80	800	0	5	2 series	++	++	+++	+++	++	++	+++	+++
82	1080	0	"	"	++	++	+++	++	+	++	++	++
				Series B 1								
91	850	+	1	1	0	+++	0	0	+	0	+	0
94	980	+	2	"	+	+++	0	0	++	0	+	0
95	940	0	3	"	0	+++	+	0	0	0	+	0
96	820	0	"	"	+	+++	+	0	++	0	0	0
61	660	0	7	"	0	+++	++	0	++	++	+	+
62	680	0	"	"	0	+++	+	0	+	+	++	+
60	820	+	14	"	0	+++	+	+	+	++	0	0
63	1110	0	"	"	0	+++	+	+	++	++	+	+

64	930	0	2	2	+	+++	+++	+	++	++	+++	+
72	920	0	"	"	+	+++	++	+	++	++	++	+
65	810	0	3	"	+++	+	+++	+	++	+++	+++	+
74	750	+	"	"	++	++	++	+	+	+++	+	+
66	700	+	7	"	+	++	++	++	+	++	++	++
71	1130	0	"	"	+	+++	+++	++	++	+++	+++	+++
100	600	0	1	3	0	+++	+++	+++	+++	+++	+++	++
104	620	0	"	"	++	++	++	++	+++	+++	++	+
98	760	0	3	"	++	++	+++	++	+	+++	+++	+++
101	680	0	"	"	+++	+	++	+	++	+	++	+
67	900	+	7	"	++	+	++	++	++	+++	+++	+++
73	980	0	"	"	++	+	+++	+++	++	+++	+++	+++
102	680	0	"	"	+	++	+	++	+	++	+	+
97	830	0	1	4	+	++	++	+	+	+	++	+
99	800	0	"	"	+	+++	++	++	+++	+++	++	+
105	985	0	3	"	++	+++	+++	+++	++	+++	+++	++
106	785	+	"	"	+	+++	+++	++	++	+++	+++	++
69	720	0	7	"	++	+	+++	+++	+	+++	+++	+++
107	860	0	"	"	+	++	+++	+++	+++	+++	+++	++
108	1000	0	"	"	+	++	++	++	++	++	+	+

Series B 2

141	1000	+	5	3	+	+++	+++	+++	+++	++++	++++	+++
128	1250	0	"	4	+++	+	+++	+	+++	+++	−	−
140	1100	0	"	"	++	++	+++	++	+++	++++	++++	+++

further influence. In the animals treated with large single doses hyperplasia took place which had reached its highest point after an initial atrophy in 14 days. This was followed by increasing atrophy, which led in most of the animals to complete atrophy.

(*d*) *Liver.*—The changes in the liver took place mainly in the capillaries, but partly also in the periportal connective tissue and around the minor vessels.

In the capillaries at first appeared increased numbers of basophilic cells, partly of the type of lymphocytes and plasma cells, partly of the type of histiocytes and reticulo-endothelial cells. Swollen Kupffer cells with intensely basophilic protoplasm were seen, which were still connected wholly with the vessel wall. Other cells also appeared which had already acquired a more rounded shape, and which retained only slight connection with the wall. Finally loose Kupffer cells were found.

Infiltrations of round cells were found in all stages of transition such as are found in the livers of normal rabbits. In larger infiltrated areas some polymorphonuclear leucocytes and phagocytes filled with pigments also were seen. As is shown in Table I, infiltrations of round cells were found in normal rabbits with some regularity, but they were never seen to such an extent as in the immunized animals (Fig. 8). Here also karyokinetic figures were found, which are very rare in normal animals. Sometimes these infiltrations lay beneath the walls of medium sized vessels (Fig. 9) and pressed the endothelium towards the lumen. Intensely basophilic cells were often observed in the lumen still in connection with the endothelium of these vessels. Apparently these were migratory cells.

The infiltrations often contained giant cells (Fig. 10). In the animals treated with large doses they appeared in such great numbers that they seemed to predominate over the other cells of these areas. They were exactly like the giant cells of the spleen, so that they need not be described again. Often they contained brown pigment. They were found mainly in the peripheral parts of the lobules, and were occasionally seen in the walls of medium sized veins.

In the animals treated with small doses the mesenchymal reactions just described, reached their greatest development in the week after the second series of injections (Table I) as in the spleen. In the animals treated with large single doses the reaction was developed intensely from the time of the second injection and remained in the same state until the end of the series. Giant cells were not seen in the livers of normal rabbits. In the animals treated with small doses giant cells were found the 4th day after the first series of injections, whereas they were seen in the animals treated with large single doses from the 7th day after the first injection. The difference between the series A and B consisted, as in the spleens, first, in a more intense reaction after large doses and second, in the fact that after small doses, the round cells predominated, whereas after large doses many more giant cells appeared.

(*e*) *Lungs.*—In the mesenchymal reactions the lungs took as important a part as the liver and spleen, if not, indeed, a greater one.

The changes took place in the vascular connective tissue. At first an increased number of cells of the type of lymphocytes and plasma cells and of the type of histiocytes and reticulo-endothelial cells appeared in the adventitial tissue of the minor vessels (Fig. 11). These intensely increased in number as was shown by many karyokinetic figures. At the same time similar cells appeared in many places in the alveolar walls. In the peribronchial tissue also a certain degree of proliferation was found. In the small vessels especially in the veins proliferation between media and intima occurred, which consisted mainly of reticular cells, but always contained varying numbers of round cells. It is remarkable, that here the media was usually preserved as a distinctly visible band (Fig. 12). The proliferation pushed the endothelium towards the lumen. The endothelium could always be clearly distinguished from the proliferation (Fig. 12). The lumina of the vessels grew more and more narrow and finally often entirely disappeared. During the progress of this reaction (Fig. 18) the alveolar walls thickened either diffusely, or in nodules, and the alveolar lumina decreased in number and size. The cellular character changed to one of reticular composition and many lattice fibres appeared. Finally the alveoli became more or less solid (Fig. 15), so that it was nearly impossible to differentiate the elements. The vessels were still mostly recognizable as solid bands. The lumina of the alveoli often entirely disappeared, and in other places were recognized merely as narrow spaces filled with alveolar epithelial cells.

Giant cells also appeared which reached large numbers, mainly in the animals treated with large doses. They too were of the Langerhans type and similar to those described in the spleen. In the alveolar walls highly basophilic round cells were also seen not only outside of the capillaries but also in their lumina. Especially large numbers of giant cells were sometimes found between media and intima of the medium sized vessels.

As is shown in Table I, such mesenchymal reactions were found in 2 normal animals and even a few giant cells were seen. But these changes were very slight. In the few animals of series A the lungs of which were examined, this reaction usually took place and giant cells appeared regularly. In the animals treated with large single doses this reaction was found with great regularity from the 7th day after the first injection and was well developed after the second injection. Many giant cells were found. In the animals of series B 2 they were also found in great numbers.

(*f*) *Adrenals.*—In the adrenals the mesenchymal reactions were observed only in animals treated with large doses. All in all they were but of slight extent.

TABLE II.

Reactions in Adrenals, Kidneys and Heart in Relation to the Time and Number of Intravenous Injections of Killed Staphylococci.

No.	Weight	*	Time after last injection	No. of injections	Adrenal		Kidney		Heart	
					Interstitial infiltration	Giant cells	Interstitial infiltration	Giant cells	Interstitial infiltration	Subendocardial infiltration
Normal animals										
	gm.									
56	380	0			+	0	0	0	0	0
54	590	0			0	0	0	0	0	0
53	750	0			0	0	0	0	0	0
55	780	0			+	0	0	0	0	0
58	1200	0			0	0	0	0	+	0
59	1240	0			0	0	0	0	0	0
51	1730	0			0	0	0	0	0	0
52	2130	0			+	0	+	0	++	0
49	2460	0			0	0	0	0	+	0
48	2740	+			0	0	++	0	+	0
50	2800	0			0	0	0	0	0	0
Series B 1										
			days							
91	850	+	1	1	0	0	+	0	0	0
94	980	+	2	"	0	0	0	0	0	0
95	940	0	3	"	0	0	0	0	0	0
96	820	0	"	"	0	0	0	0	0	0
61	660	0	7	"	0	0	++	0	0	0
62	680	0	"	"	0	0	0	0	0	0
60	820	+	14	"	0	0	0	0	0	0
63	1110	0	"	"	0	0	0	0	+	0
64	930	0	1	2	0	0	0	0	+	0
72	920	0	"	"	0	0	+	0	0	0
65	810	0	3	"	0	0	+	0	0	0
74	750	+	"	"	+	0	+	0	0	0
66	700	+	7	"	+	0	0	0	0	0
71	1130	0	"	"	++	0	+	+	++	++
100	600	0	1	3	0	0	0	0	+	0
104	620	0	"	"	0	0	0	0	++	+
98	760	0	3	"	++	+	0	0	+	+
101	680	0	"	"	0	0	0	0	0	0
67	900	+	7	"	+	0	+	+	++	0
73	980	0	"	"	++	0	++	0	++	0
102	680	0	"	"	++	0	++	0	0	+

* Moderate coccidiosis, echinococcus or tuberculosis.

TABLE II.—*Concluded.*

No.	Weight	*	Time after last injection	No. of injections	Adrenal		Kidney		Heart	
					Interstitial infiltration	Giant cells	Interstitial infiltration	Giant cells	Interstitial infiltration	Subendocardial infiltration
Series B 1—*Concluded.*										
	gm.		*days*							
97	830	0	1	4	0	0	0	0	0	0
99	800	0	"	"	0	0	0	0	0	0
105	985	0	3	"	+++	+	0	0	++	0
106	785	+	"	"	++	+	++	0	+	0
69	720	0	7	"	+	0	+	+	+++	0
107	860	0	"	"	+++	++	0	0	+	0
108	1000	0	"	"	+	0	0	0	0	0
Series B 2										
141	1000	+	5	3	++	++	+	+	−	−
128	1250	0	"	"	0	0	−	−	−	−
140	1100	0	"	"	++	+	++	++	−	−

They were similar to those seen in the liver and took place mainly in the capillaries. In a part of the animals increased numbers of intensely basophilic cells were found in the capillaries mainly in those parts of the cortex next to the medulla. Later infiltrations were found (Fig. 13), which contained even giant cells (Fig. 14). While in 3 normal animals only, a few infiltrations composed for the most part of slightly basophilic cells were found (Table II), they were seen in most animals treated with large doses from the time of the second injection. Giant cells were missing entirely in normal animals and were observed in series B only in 6 instances.

(*g*) *Kidneys.*—In the kidneys of the animals treated with large doses interstitial accumulations of round cells mixed with polymorphonuclear leucocytes were frequently observed, more frequently than in normal animals (Table II). It seems that these changes were partly caused by the treatment. In 5 cases of series B giant cells were found in the glomeruli (Fig. 16), and seemed to be derived from the covering cells of the loops. In normal animals no giant cells were seen.

(*h*) *Heart.*—The changes in the heart course were like those in the kidneys and the adrenals. Interstitial accumulations of round cells

were found in normal rabbits as a rule only in advanced age, whereas in the animals of series B 1 there was a distinct increase during the injections (Table II). In Fig. 17 such an infiltration is shown. Giant cells were not found. In 4 of the cases treated with large doses subendocardial accumulations of round cells were also seen, which were similar to those in the vessels of the liver.

DISCUSSION.

The histological changes which appeared in our animals after intravenous injections of killed staphylococci, took place on the one hand, in certain lymphatic tissues and in the thymus, on the other, in the vascular connective tissue. As a rule, after small doses the changes in the lymphatic organs predominated, but after large doses those in the vascular connective tissue were the outstanding ones.

1. Changes of the Lymphatic Tissue and of the Thymus.

The changes in the lymphatic tissue affected especially the spleen and the peripheral lymph nodes (cervical, axillary and popliteal lymph nodes). The weight of the spleen increased up to 250 per cent. Histologically an enormous increase in number and partly in size of Flemming's secondary nodules took place. The peripheral lymph nodes increased in weight up to 120 per cent. Microscopically this increase was due to lymphatic hyperplasia. In certain stages (14 days after the first injection) there occurred in both series an increase in weight of the thymus up to 100 per cent. If there is taken into consideration furthermore the fact that in the heart and other organs large infiltrations of round cells were found and also that the animals succumbed readily during the experiments to intercurrent diseases, it is entirely credible that a status thymicolymphaticus was produced.

Because the status thymicolymphaticus lately has attracted new interest, it will be well to add some brief remarks on it since my study throws a new light on its etiology and significance.

On the etiology of the status thymicolymphaticus there exist three theories (literature in Thomas (23)). Some authors still maintain the theory of Paltauf (24), which states that this disease is a constitutional anomaly. Others, like Lubarsch (25), doubt whether the status thymicolymphaticus is related to any special predisposition and

wonder whether it must not rather be looked upon as an effect developed in consequence of certain toxins which in part enter the body from the outside, and in part originate within it, or perhaps both. Others further believe that the status is the consequence of increased nutrition. Jaffe (26–28) and Marine (29–30) suppose on account of their well known adrenalectomy experiments that the status thymicolymphaticus is a direct result of the hypofunction of the adrenals and that normally the adrenals suppress or exert a regulating effect upon the thymus and the lymphatic tissue.

Against the last theory may be advanced the evidence that in status thymicolymphaticus the adrenals are usually absolutely normal, and that on the other hand, many cases of atrophy of adrenals are encountered which do not exhibit the status thymicolymphaticus. In my experiments also no relation between the weights of the thymus and of the adrenals was found. Finally, this theory cannot explain an observation of Schridde (31), namely, that in status lymphaticus local hyperplasia of lymph nodes only is usually found. Because this point seems to be so important for an understanding of this disease, I cite briefly 3 examples, which were observed during the last year.

1. In a woman 31 years old (A, No. 284), who died of Addison's disease, primary, essential or idiopathic atrophy of the adrenals was found at autopsy. The organs weighed together 4 gm. The thymus weighed 50 gm. Hyperplasia of the lymph nodes occurred mainly around the intestine; the mucous membrane of the duodenum was penetrated by numerous lymph nodules. The microscopical examination showed, that the villi of the duodenum were unusually rich in round cells.

2. In a man 24 years old (A, No. 300), who had been suffering from chronic mitral stenosis, and who died suddenly of fibrillation of the heart, a thymus of 40 gm. was found. The lymphatic hyperplasia occurred mainly in the neck and mediastinum. The tonsils had been removed 5 weeks before death because of chronic inflammation. The adrenals weighed together 9 gm. and were of normal size.

3. In a medium sized cat which died suddenly at the beginning of an ether narcosis, an enormous thymus of 6½ gm. was found, a weight, which surpasses the highest weight found by Friedleben (32) in normal cats, stated to be about 4 gm. (55 gr.). The status thymicolymphaticus was a more universal one, but was noticed especially around the intestine. The lower end of the small intestine and the colon were studded with lymph nodules. Microscopically numerous round cells and polymorphonuclear leucocytes were found in the wall. The adrenals appeared to be normal.

The common feature of these 3 cases is the local collection of the hyperplastic lymph nodes at the sites which according to Schridde (31) are typical: around the intestine and the neck. The corresponding parts of the mucous membrane of the intestine like that of the throat showed marked signs of chronic inflammation. If Marine's theory were correct uniformly distributed hyperplasia of all lymph nodes should be expected. Local hyperplasia speaks much more for the theory of Lubarsch. I assume with Lubarsch (25) that the status thymicolymphaticus usually is caused by certain "toxins," or by bacteria and their products and that in atrophy of the adrenals or after adrenalectomy the resistance of the body against such products is reduced. The adrenals do not exert a suppressing or regulating effect upon the lymphatic tissue, but rather work independently of the lymphatic tissue in destroying these products. The appearance of local hyperplasia seems to indicate merely the place of entrance of the "toxin."

The question, whether there is a constitutional form of the status thymicolymphaticus will not be discussed.

2. Lymphocytosis and Lymphatic Tissue.

The lymphocytosis which occurred in these experiments, was parallel in time and in amount with the hyperplasia of the peripheral lymph nodes. In the lymph nodes an increase in size and number mainly of the pseudo-secondary nodules appeared. The peripheral lymph nodes were in all probability responsible for the lymphocytosis and the pseudo-secondary nodules were its place of origin as previously discussed (1). The lymphocytosis can hardly be brought into direct relation with Flemming's secondary nodules in the spleen. The highest development first attained of Flemming's secondary nodules in the spleen coincided in both series with the highest point of the lymphocytosis. But whereas later the numbers of lymphocytes decreased to normal, Flemming's secondary nodules reached a new high point of development which quite equalled the first one after each single injection or series of injections (*cf.* Text-figs. 1 and 2 with Table I). A relation seems therefore to exist between injections and Flemming's secondary nodules in the spleen like that which exists in infected animals (1) between subcutaneous abscesses and Flemming's secondary nodules.

3. Mesenchymal Reactions.

The mesenchymal reactions after intravenous injection of killed as well as of various living bacteria, described by Oeller (2–5), Siegmund (6–8) and others seem from their descriptions to be of two different kinds. On the one hand, Oeller described as a general cellular process the appearance of numerous polymorphonuclear leucocytes in the capillaries of liver, lungs and other organs which are derived locally he thinks from endothelial cells. This he calls an endothelial reaction. On the other hand, he described an adventitial reaction in which large adventitial and interstitial lymphoid and plasma cellular proliferations make their appearance in the organs. Siegmund finally described granulomas arising in the intima of vessels. These authors except Oeller (5) agree in so far as they state that all these reactions appear only after repeated injections and must be looked upon as due to the reinjection. They are said to appear as soon as a few minutes after reinjection and to disappear quickly afterwards. On the basis of these investigations they have proposed a cellular theory of the immunizing process.

These authors have been opposed by Gerlach and his associates (17–19) as well as by Seemann (14–16); both maintain that these reactions do not take place at all, but represent normal pictures. They deny a participation of the lungs in these processes of any significant proportion. The failure of Gerlach and Seemann to notice these changes may probably be explained partly by the small number of their experiments, but partly also by their brief duration.

The studies here reported show that after intravenous injections with killed staphylococci enormous mesenchymal reactions appear. These are reactions partly of a lymphocytic and plasma cell nature, and partly the result of the proliferation of histiocytes and reticuloendothelial cells which occur in vascular connective tissue. In animals treated with large doses especially numerous giant cells appeared. This finding is here described for the first time.* The lungs, spleen

* After this work was completed Dr. Bach reported in a meeting of the American Pathological Society somewhat similar alterations apparently in the lungs and livers of rabbits following prolonged immunization with non-hemolytic streptococci.

and liver take the largest part in these processes, whereas similar changes, though only of slight degree, are found also in the adrenals, kidneys and heart, but only after large doses. In the animals treated with small doses a reaction of small cells prevailed, whereas after large doses giant cell formation predominated.

This reaction appears to be the same as that which both Oeller and Siegmund described. Oeller found the lesions he described chiefly in the adventitial tissue. Those which Siegmund saw were chiefly in the intima. Oeller accordingly described these as "adventitial reaction" and Siegmund as "intima granuloma." Because of the insufficiency of my experiments I cannot discuss Oeller's "endothelial reaction."

The adventitial reaction of Oeller is not necessarily an effect of reinjection, as is generally supposed, because I found it, as Oeller did also partially (5), soon after the first injection (Table I), if a dose sufficiently large was given and the animals were killed 1 to 2 weeks later. After repeated injections the reaction appears to be greater, but this effect may probably be cumulative. As the reaction was most striking in animals injected with large doses, and these animals were found to produce agglutinins in only very low concentrations, as the controls showed, this reaction must be regarded as a tissue response of the organism to the bacteria or their products. We may call it a primary reaction rather than a prolonged immunizing process.

SUMMARY.

In 87 rabbits, some of which received intravenously at various intervals small, and some large, doses of killed staphylococci, the following findings were obtained.

1. There occurred a hyperplasia of lymph nodes, spleen and thymus, that is to say, a status thymicolymphaticus. This phenomenon is explained as due to immediate local irritation caused by bacteria and their products and by certain "toxins" partly of exogenous, partly of endogenous, origin.

2. The lymphocytosis which appeared was parallel in time and degree with the hyperplasia of the peripheral lymph nodes (axillary, popliteal and cervical lymph nodes) and probably originated in the pseudo-secondary nodules of these nodes.

3. There occurred intense mesenchymal reaction in the vascular connective tissue of the lungs, liver and spleen and after large doses slighter ones in adrenals, kidneys and heart. These reactions correspond with Oeller's adventitial reactions and Siegmund's intima proliferations. In the interstitial tissue of these organs as well as in the walls of the minor vessels proliferations of cells, partly of the type of lymphocytes and plasma cells, partly of that of histiocytes and reticulo-endothelial cells, appeared, which, after large doses, were mixed with many giant cells of Langerhans' type. After small doses lymphocytes and plasma cells predominated, after large doses histiocytes and reticulo-endothelial cells. Because these reactions occurred immediately after the first injection, they can be regarded as primary reactions of the organism to bacteria and their products.

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EXPLANATION OF PLATES.

PLATE 27.

FIG. 1. Axillary lymph node (R 136). Increase in number and size of the pseudo-secondary nodules (*a*). At the margin of the nodes solid (*d*) and Flemming's secondary nodules (*b* and *c*) are seen. Iron-hematoxylin-eosin × 16.

FIG. 2. Spleen (R 177). Numerous Flemming's secondary nodules are seen. They contain marginal (*a*) and outer zones (*b*). Iron-hematoxylin-eosin × 32.

FIG. 3. Spleen (R 69). Mesenchymal reaction. A number of giant cells (*a*) are seen. Iron-hematoxylin-eosin × 200.

FIG. 4. Spleen (R 64). Mesenchymal reaction. Proliferation of basophilic round cells around the minor vessels (*a*). Methyl green-pyronine × 110.

PLATE 28.

FIG. 5. Thymus (R 69). Atrophy. Iron-hematoxylin-eosin × 22.

FIG. 6. Thymus (R 58). The picture of a normal thymus. Methylene blue-eosin × 22.

FIG. 7. Thymus (R 71). Hyperplasia of the lobules, as is seen by their rounded shape and the straightness of their septa. Methylene blue-eosin × 22.

FIG. 8. Liver (R 43). Mesenchymal reaction. Increased round cellular infiltrations in the periportal connective tissue. Iron-hematoxylin-eosin × 110.

FIG. 9. Liver (R 92). Mesenchymal reaction. Round cellular infiltration in the wall of a vein. Iron-hematoxylin-eosin × 220.

PLATE 29.

FIG. 10. Liver (R 87). Mesenchymal reaction. Giant cells in the periphery of the lobules. Methyl green-pyronine × 200.

FIG. 11. Lung (R 64). Mesenchymal reaction. Adventitial proliferation of a minor vessel. Iron-hematoxylin-eosin × 110.

FIG. 12. Lung (R 66). Mesenchymal reaction. Proliferation of reticular cells between media (*a*) and intima. The endothelial cells are distinctly seen (*b*). Iron-hematoxylin-eosin × 300.

FIG. 13. Adrenal (R 102). Round cellular infiltrations in the cortex. Methylene blue-eosin × 300.

FIG. 14. Adrenal (R 141). Giant cells in the cortex. Methylene blue-eosin × 300.

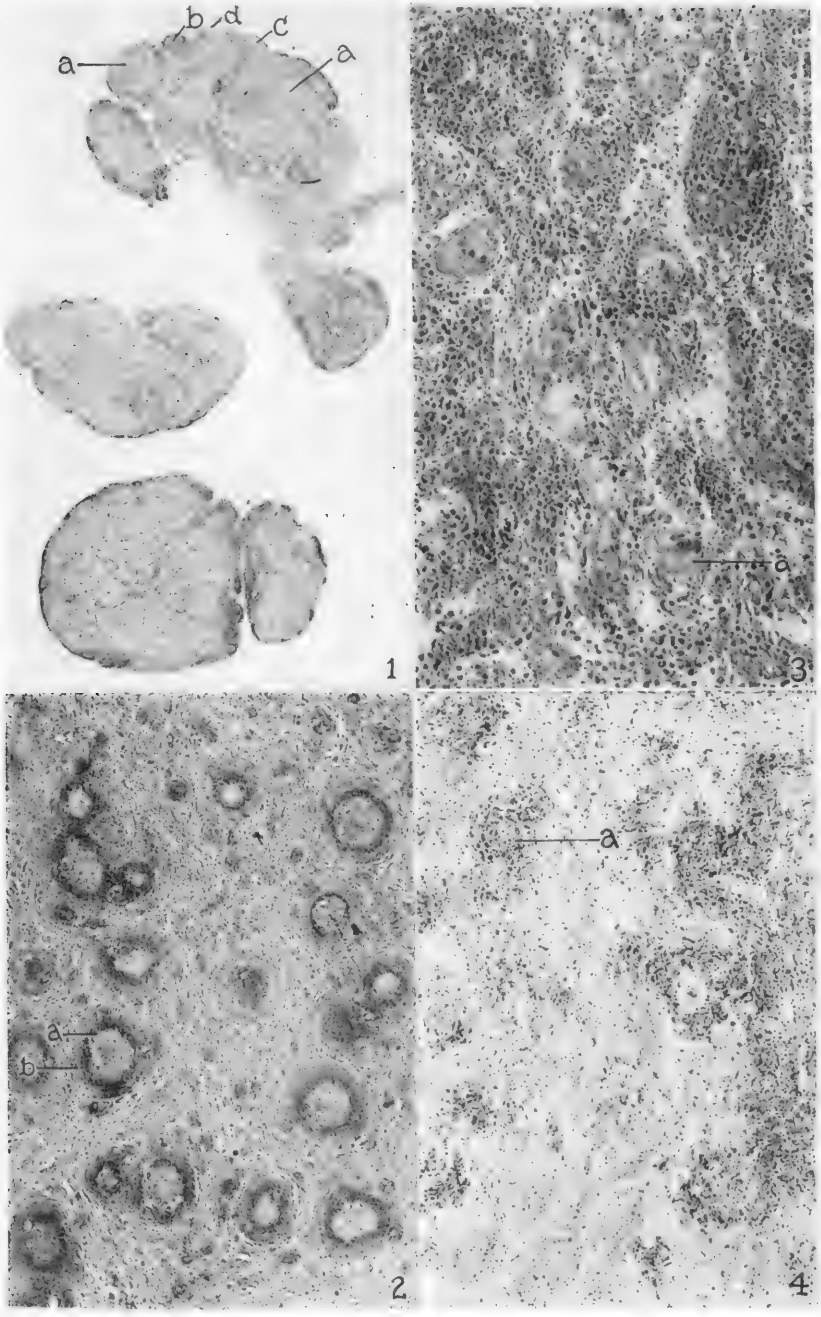
PLATE 30.

FIG. 15. Lung (R 141). Intense mesenchymal reaction. (*a*) Artery, (*b*) vein. Iron-hematoxylin-eosin × 22.

FIG. 16. Kidney (R 140). Mesenchymal reaction. Giant cellular formation in a glomerulus. Iron-hematoxylin-eosin × 300.

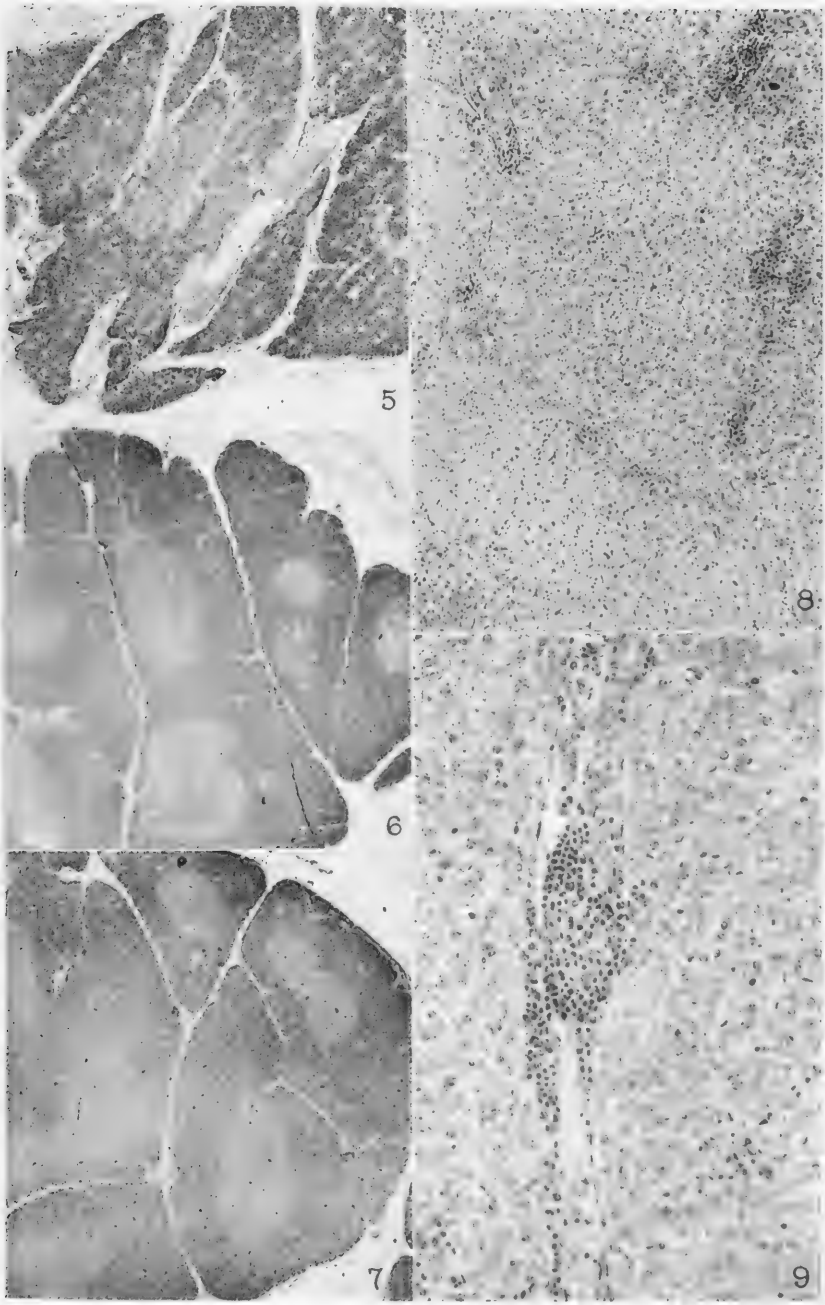
FIG. 17. (R 69). Round cellular infiltration in the myocardium. Iron-hematoxylin-eosin × 300.

FIG. 18. Lung (R 70). Advanced mesenchymal reaction. Obliteration of a vein (*a*). Thickenings of the alveolar walls (*b*). Iron-hematoxylin-eosin × 30.



Photographed by Louis Schmidt.

(Ehrich: Lymphatic tissue. IV.)



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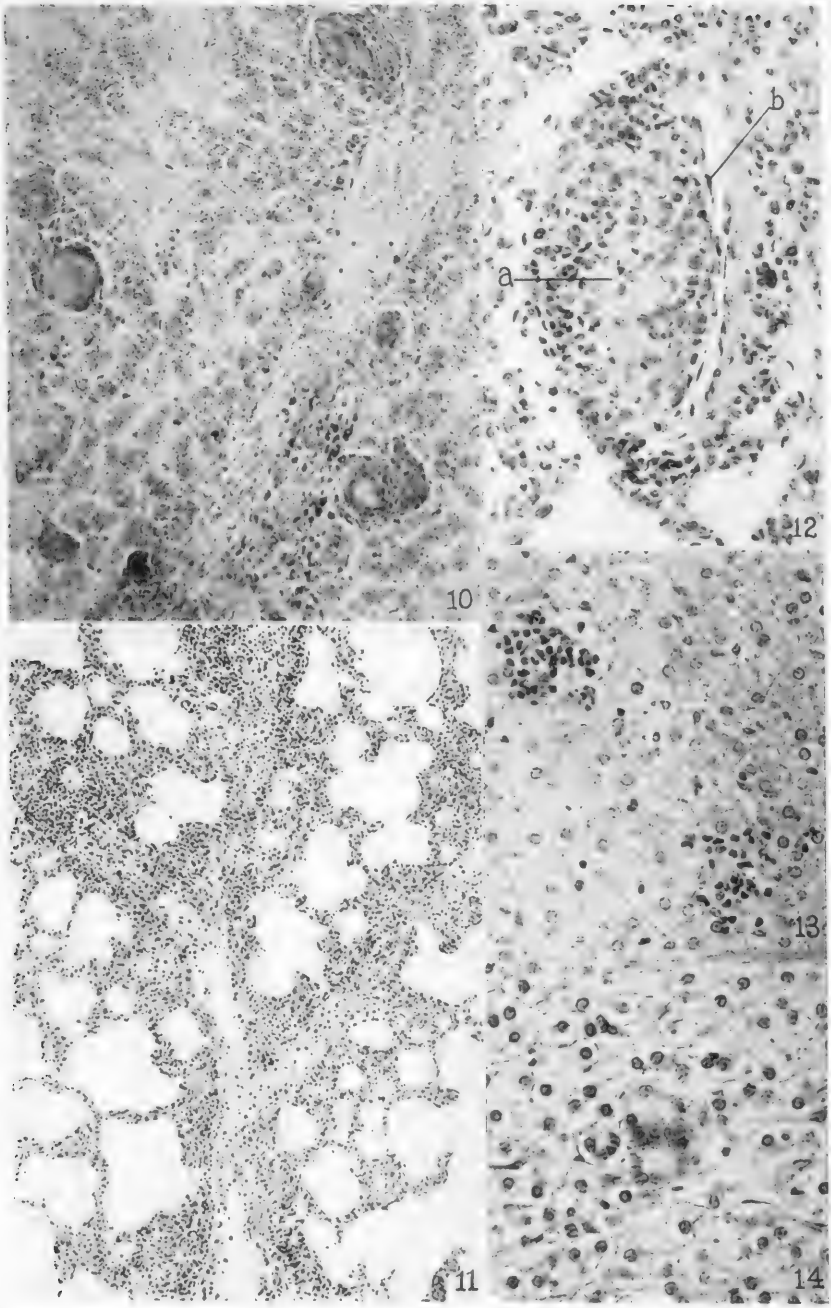

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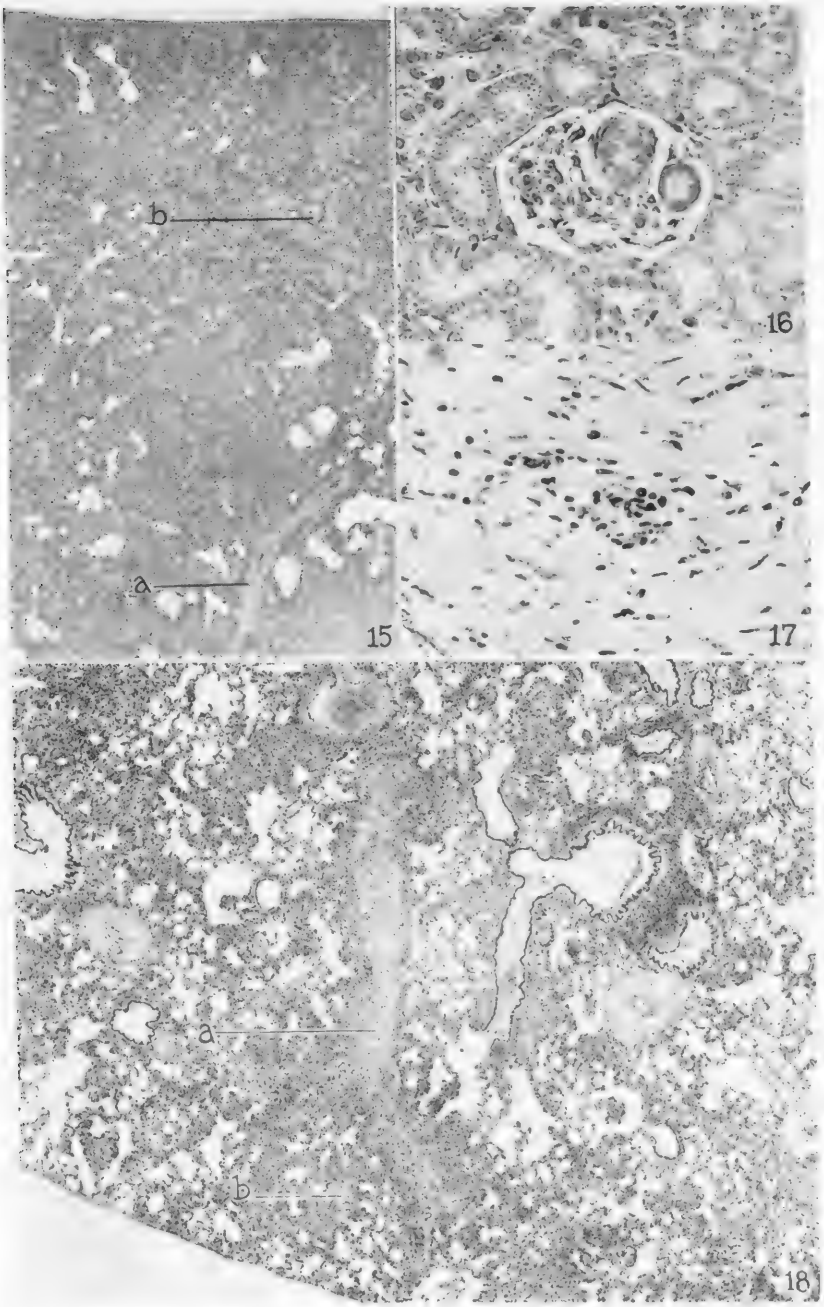

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(Ehrich: Lymphatic tissue. IV.)

JOHNS HOPKINS UNIV. OF WICH.

THE DEVELOPMENT OF THE CHRONIC NEPHRITIS INDUCED IN THE DOG BY URANIUM NITRATE. A FUNCTIONAL AND PATHOLOGICAL STUDY WITH OBSERVATIONS ON THE FORMATION OF URINE BY THE ALTERED KIDNEYS.*

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Plates 31 to 33.

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The object of the following study which has extended over a period of nearly 6 years has been to provoke in the dog by a single severe injury reparative processes involving the various histological elements of the kidney which may have definite functional expressions and at various stages of the acute injury and during the processes of repair which finally terminate in a chronic nephritis, to study the microscopic alterations in renal structure as correlated with the functional disturbance expressed by changes in the blood and urine.

No general review dealing with acute uranium nephritis will be attempted in this study. A detailed review (1) of the subject has been previously made and is available. The question of the selective affinity of uranium for the different histological elements of the kidney, especially in connection with the present experiments, warrants an analysis.

Leconte (2), and much later Richter (3, 4), observed the ability of uranium salts to induce an acute renal injury. Schirokauer (5) and Heineke and Myerstein (6) induced both vascular and epithelial injuries from uranium and noticed the occurrence of an edema. Much later than this Christian (7), Christian, Smith and Walker (8) and Christian and O'Hare (9) in their experiments with rabbits noted the predominance of the renal injury from uranium in the tubules

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and also described structural changes in the glomeruli in the form of an acute degeneration characterized by the formation of hyaline droplets. In a later paper by O'Hare (10), in which dogs were used in place of rabbits, he was unable to induce the hyaline changes in the glomerular capillaries but noted the marked selective action of the poison for the renal tubules. Pearce (11) in his early work with dogs classes uranium along with the chromates and considers the early damage to the kidney to be largely confined to the convoluted tubules. In the initial studies of Schlayer and Hedinger (12) which were followed by those of Pearce, Hill and Eisenbrey (13) on the vascular response of the kidney acutely nephropathic from various agents, the localization of the nephrotoxic action of uranium to the convoluted tubules was emphasized, and the fact established that with such an injury the vasoconstrictor and vasodilator response of the renal vessels remained unaffected. In several studies (14–17) from this laboratory both of a functional and anatomical character similar conclusions were made relative to the localization of the uranium action during the early stages of the intoxication. In 1912 Suzuki (18) and later Mitamura (19) working in Aschoff's laboratory demonstrated by a process of intravital staining, not only the affinity uranium has for the proximal convoluted tubule, but for different segments of these tubules. In these researches the resistance of certain types of regenerated epithelium was noted and the changes of repair discussed. In later papers by Oliver (20, 21) and by MacNider (22), functional changes have been considered in relation to the acute tubular injury and during the subsequent stages of early epithelial repair. From the anatomical studies to which reference has been made, as well as those which include superficial functional observations, it would appear as established for the dog that uranium induces an acute injury which is predominantly tubular in character and that the vascular injury in the early stages, if it occurs at all, is largely functional. The main histological evidence for the acute glomerular injury may only develop much later, and consists in the changes of repair which arise in the glomerular capillaries and their surrounding capsule.

Likely on account of the frequency with which the acute renal injury from uranium has returned to a functional normal the substance has not been extensively used to induce a chronic nephritis. In 1909 Dickson (23, 24) by the use of uranium over long periods in guinea pigs, rabbits and dogs was able to induce a chronic renal injury with changes in the type of tubular epithelium, glomerular changes and an overgrowth of connective tissue. These studies were followed by those of Smith (25), O'Hare (26), Frothingham, Fitz, Folin and Dennis (27) and those of MacNider and Oliver previously referred to. Very recently Hunter (28) has studied the anatomical changes incident to repair in a uranium nephritis in rabbits and has continued these studies to the stage of chronic renal injury.

Technique of Experiments.

Eighty-six dogs were employed in this study. Fourteen of the animals during the period allowed for preliminary observations were found to be naturally nephro-

pathic and were excluded from the series. Seventy-two of the animals showed no abnormality and were used for experimental purposes. Depending upon the reaction of these animals to a constant quantity of uranium nitrate per kilo when given subcutaneously in terms of their ability to withstand the acute intoxication or to develop processes of repair, they may be divided into three groups. In previous studies (29, 30) the observation has been made in connection with the susceptibility of dogs to the general anesthetics, the ability to establish protection against such bodies, as well as concerning the nephrotoxic action of uranium nitrate, that the age of the animal, regardless of the weight, very largely determined the severity of the animal's reaction both pathologically and functionally. The same factor, that of age, has more recently been found to exert its influence during gestation in determining the incidence of the development of a disturbance in the acid-base equilibrium of the blood in pregnant dogs (31, 32). On this account, in the series of dogs now under consideration animals were selected in widely separated age groups with the object in view of modifying the intensity of the acute degenerative phase of the kidneys' reaction to uranium. Group I is represented by twenty-one dogs which were over 7 years of age. These animals either died during the acute stage of the nephritic process or were killed for purposes of study. Group II comprises fifteen dogs which, after having developed a severe acute nephritis, returned to a complete functional normal. Group III is made up of thirty-six animals which, after having developed an acute nephritis failed to make a functional adjustment back to the normal, but were left with such changes in the kidneys and gave evidence of such functional alterations in the blood and urine that they have been classed as animals with a chronic nephritis. The dogs in Groups II and III were between 1 and 2 years of age. Only these relatively young animals in the last two groups were able after the development of an acute nephritis to return to a functional normal or to a state of pathological repair with the acquisition of a chronic nephritis.

During the various periods of study the animals were kept in metabolism cages, given 500 cc. of water daily by stomach tube and allowed an unrestricted mixed diet of bread with butter, milk and meat. Catheterized specimens of urine were examined for albumin by the usual heat and acid test and in addition by the very delicate contact reagent of Mayer. Centrifugalized specimens were studied for casts. The immediate functional capacity of the kidneys during a 2 hour period was determined by the phenolsulphonephthalein test of Rowntree and Geraghty (33). Evidence of a renal dysfunction in terms of retention was ascertained by blood urea determinations by the method of Marshall (34) as modified by Van Slyke and Cullen (35) and by non-protein nitrogen and creatinine determinations by the methods of Folin and Wu (36). Alkali reserve determinations of the blood and the volume per cent of carbon dioxide were used as indices of a disturbance in the acid-base equilibrium of the blood and were made by the methods of Marriott (37) and Van Slyke and Cullen (38).

The animals were given one subcutaneous injection of 4 mg. of uranium nitrate per kilo. Observations subsequent to the injection were in a measure determined

TABLE I.

Group I. Experiment 50. Duration of Experiment, 13 Days.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphonephthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations	10.5 kg. over 7 yrs.	0	500	512	0	0	0	0	75	8.1	12	27	1.7	Kidneys show diffuse congestion. When capsule is incised swollen cortex bulges into incision. Capsule not adherent. On section there is disproportion in volume between cortical and medullary portions. Tissue cuts without resistance. Glomeruli prominent
10/24/26														
10/30/26		4	500											
11/2/26 3rd day of intoxication		0	500	1400	1.5	Present	Present	Numerous granular. Few hyaline	35	7.9	25	29	2.2	
11/4/26 5th day of intoxication		0	500	1440	2.5	Present	Present	Numerous granular casts	Trace	7.7	62.5	120	4.0	Histological study shows glomerular capillaries distended with blood. No subcapsular or intertubular exudates. Glomerular endothelium prominent. Nuclei stain well
11/6/26 7th day of intoxication		0	500	511	1	Present	0	Numerous granular casts	0	7.7	120	255	6.1	
11/8/26 9th day of intoxication		0	500	355	0.5	1.0	0	No change	Trace	7.75 convulsions	150	255	6.	Epithelium of convoluted tubules edematous and in areas necrotic. Nuclei stain imper-

11/12/26 13th day of intoxication		0	500	291	0.25	Trace	0	No change	5	7.85 convulsions	150	216	5.3	fectly or have disappeared. Epithelium of Henle's loops shows no evidence of injury. Casts present in tubules. No evidence of epithelial regeneration in damaged convoluted tubules

by the severity of the animal's reaction and by the findings in the urine and blood which made it desirable to terminate a given experiment for the purpose of checking the functional response with the then existing pathological changes in the kidneys.

Group I. Animals with an Acute Nephritis Which either Died as a Result of the Process or Were Terminated during Its Course.

Twenty-one dogs are included in this group. Table I gives in detail the course of the nephritis in Experiment 50. None of the dogs in this older group survived the acute injury longer than 14 days. They were used at intervals of 2 or 3 days from the commencement of the nephritis to the 14th day for purposes of histological study in connection with the functional findings.

By the commencement of the 3rd day following the use of uranium the animals became polyuric with a urine containing albumin which varied in amount from 1.5 to 4.6 gm. per liter. The urine contained both glucose and acetone and coarsely granular casts. This type of cast without a transition to the hyaline type persists throughout the acute nephritis. In Experiment 50, Table I, Group I, the urine increased from 512 cc. to 1440 cc. At the same time that such changes took place in the volume output of urine, there occurred a marked reduction in the elimination of phenolsulphonephthalein, a depletion in the reserve alkali of the blood and a beginning retention of urea, non-protein nitrogen and creatinine. These changes increased in severity throughout the course of the experiments in this group. In the animal of Experiment 50, the elimination of phenolsulphonephthalein was reduced on the 3rd day of the nephritis from the normal of 75 per cent to 35 per cent the reserve alkali of the blood from the normal of 8.1 to 7.9 with a commencing retention of urea, non-protein nitrogen and creatinine. On the 7th day of the nephritis there was a total output of urine of 511 cc., which contained 1 gm. of albumin per liter, glucose and numerous broad, granular casts. There was no elimination during a 2 hour period of phenolsulphonephthalein. The reserve alkali of the blood was reduced to 7.7; there was a retention of blood urea of 120 mg., non-protein nitrogen of 255 mg. and of creatinine of 6.1 mg. On the 13th and terminal day of the nephritis with an intake of 500 cc. of water which was vomited, there was an output of 291 cc. of urine which contained albumin with no apparent change in the number or character of the casts. Phenolsulphonephthalein was present as a trace and the reserve alkali of the blood was 7.85. There was a retention of 150 mg. of urea, 216 mg. of non-protein nitrogen and 5.3 mg. of creatinine. The animal died in convulsions on this day of the experiment. In Experiment 11, on the 2nd day of the nephritis, the animal formed 916 cc. of urine, which contained granular casts and 3.8 gm. of albumin per liter. The elimination of phenosulphonephthalein was reduced from the normal of 82 per cent to 14 per cent, the reserve alkali to 7.8 from a normal of 8.1. There was a

retention of blood urea of 71 mg., non-protein nitrogen of 168 mg. and creatinine of 4.2 mg. On the 3rd day of the nephritis there was a volume output of 1086 cc. of urine with a negative elimination of the dye, a reserve alkali of 7.6, a retention of urea of 112 mg., non-protein nitrogen of 271 mg. and creatinine 6.7 mg. The experiment was terminated to study the early renal injury in connection with its functional expression.

The autopsies on the animals in the various groups, unless otherwise noted, were performed immediately after death or during the death process. Other than the total output of urine and its contents, the final data obtained from each animal are an expression of the animal's functional response within an hour of its death.

The kidneys of the animal of Experiment 50 were swollen. The capsule was not adherent. On section there was a marked congestion, especially of the medulla. The glomeruli were prominent. The histological study showed the glomerula capillaries distended with blood. Thrombosis of these vessels was not observed. There were no subcapsular or intertubular exudates. The capillary endothelium was prominent with deeply staining nuclei. There was no hyperplasia of the capsular epithelium and no evidence of glomerular or periglomerular connective tissue formation. The severity of the changes in the convoluted tubule epithelium and its attempts at repair showed some variation in the respective animals, the amount depending upon the duration of the acute nephritis. The vascular pathology remained constant in the animals of the group. In the animal of Experiment 50, the epithelium of the proximal convoluted tubules was edematous, vacuolated and in areas necrotic. The nuclei appeared as shadows or had fragmented. Endothelial leucocytes were present in the areas of necrosis. The epithelium of the descending and ascending limbs of Henle's loops appeared normal. In four animals of this group obtained for autopsy between the 4th and 10th days of the nephritis, occasional mitotic and premitotic figures were found in the degenerated material of the convoluted tubules. These changes indicative of an attempt at repair were not observed in animals after the 10th day of the injury (Fig. 1).

Conclusions for Group I.

1. The animals of the group, when given uranium nitrate under the conditions of age and dosage as enumerated, developed an acute nephritis characterized by an early and severe injury to the proximal convoluted tubule epithelium and with but slight evidence of repair to such cells. The associated glomerular injury is characterized by the absence of any marked structural change other than a prominence of

the capillary endothelium and an engorgement of these vessels with blood.

2. The functional expression of this pathology consisted in the animal's becoming polyuric with an albuminous urine containing casts. If the intake of water could be maintained, the animals persisted as long as 14 days in a polyuric state, the output of urine equaling or exceeding the fluid intake. Associated with these changes in the volume output of urine there developed with the commencement of the epithelial injury, at a time when there was no structural evidence of glomerular injury, and progressed as the epithelial injury became intensified, a reduction in the elimination of phenolsulphonephthalein, a depletion in the reserve alkali of the blood and a marked retention of urea, non-protein nitrogen and creatinine. The mechanism concerned with water output becomes hyperactive, while that concerned with dye elimination and the elimination of certain endogenous waste products becomes progressively inactive.

Group II. Animals that, following the Development of an Acute Nephritis, Return to a Complete Functional Normal.

Fifteen of the seventy-two dogs which were given an acute nephritis from uranium were able to effect such changes of repair in the kidney that they returned to a state of normal renal function. The animals of the series which were able to make this functional readjustment were young dogs between 1 and 2 years of age. The technique of the experiments used in this group was identical with that employed in Group I. For this reason the animals in Group I serve, in so far as the acute nephritic process is concerned, as control experiments for the animals in Group II. In order to further establish as a fact that the animals of Group II which returned to a functional normal had in the early stages of the nephritis developed a renal injury similar in character to the animals of Group I, the following procedure was undertaken. During the acute phase of the nephritis in four of the animals when there was the usual correlation between the acute process and diminished renal function, the animals were protected against the anesthetic by a solution of glucose (39) and under a light morphine-ether anesthesia a wedge-shaped piece of tissue was removed from the upper pole of the left kidney. The animals recovered from the surgical

interference and finally returned to a functional normal. The pathological changes observed in this material were similar in character and localization, though not in intensity, to the changes previously described as occurring in the kidneys of the animals of Group I during an acute nephritis. The acute changes in the glomeruli showed no variation from those described for the previous group. There was a decided variation in the degree of injury to the proximal convoluted tubule cells. This observation as well as the type of repair likely explains the great variation found to exist in the rapidity of the recovery of different animals of the group. The earliest return to a state of functional normal was on the 19th day following the commencement of the nephritis, while the latest return to such a state was in the 7th month following the acute injury. Between these two extremes the remaining animals were used for study at intervals varying from 1 month to 8 months after the development of the acute nephritis.

Following the use of uranium in this group of animals the functional disturbance was similar in character though less severe than in the dogs of Group I.

The animal of Experiment 27, Table I, Group II, on the 2nd day of the nephritis had become polyuric with an output of 960 cc. of urine. The urine contained 1.4 gm. of albumin per liter, a trace of acetone and numerous granular casts. The elimination of phenolsulphonephthalein was reduced from 72 per cent to 60 per cent and the reserve alkali of the blood from 8.1 to 7.95. There was no retention of urea, non-protein nitrogen or creatinine. The lack of retention of these bodies is of interest in connection with the high percentage elimination of the dye. On the 6th day of the nephritis the functional disturbance was at its height. The animal had a volume output of 1134 cc. of urine which contained 2 gm. of albumin per liter and with an increase in the number of granular casts. The elimination of phenolsulphonephthalein was reduced to 15 per cent and the reserve alkali of the blood was depleted to 7.85. There was a retention of 60 mg. of urea, 133 mg. of non-protein nitrogen and 4 mg. of creatinine. At this stage of the acute nephritis the animal was anesthetized and kidney tissue removed to check the histological changes in the acute state with the processes of repair should the animal later return to a functional normal or develop a chronic nephritis. On the 11th day of the nephritis there was definite evidence of functional improvement. The animal remained polyuric with an output of 860 cc. of urine which contained 1.2 gm. of albumin, an increase in the number of hyaline casts and a decrease in the number of coarsely granular casts. The phenolsulphonephthalein elimination had increased to 30 per cent and the reserve alkali to 7.95. The retention of urea

TABLE I.

Group II. Experiment 27. Duration of Experiment, 19 Days.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphonephthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations 11/10/25	7.8 kg. 1–2 yrs.	0	500	435	0	0	0	0	72	8.1	15	30	1.2	Kidneys normal in both color and size. Capsule not adherent. Kidney tissue cuts without resistance. Normal relationship in volume between cortex and medulla. Glomeruli not prominent. No evidence of congestion
11/10/25		4	500											
11/12/25 2nd day of intoxication		0	500	960	1.4	0	Trace	Few hyaline. Many granular	60	7.95	15	30	1.1	
11/16/25 6th day of intoxication	Kidney tissue removed	0	500	1134	2.0	Trace	Trace	Few hyaline. Many granular	15	7.85	60	133	4.	Microscopic study shows many glomeruli to be increased in size. Capillary walls prominent. Connective tissue cells present between capillary loops. In some areas loops have become obliterated, in others, distended with blood. Capsular epithelium prominent and rarely hyperplastic
11/21/25 11th day of intoxication		0	500	860	1.2	0	0	Increase in hyaline. Few granular	30	7.95	34	70	3.	
11/23/25 13th day of intoxication		0	500	732	Trace	0	0	Few hyaline casts	60	8.1	16	33	1.7	Epithelium of convoluted tubules shows advanced stage of repair. Mitotic figures present in many of these specialized cells. In other cells in which process of repair has been completed cytoplasm stains uniformly and nuclei hyperchromatic. No increase in intertubular connective tissue
11/29/25 19th day of intoxication		0	500	468	0	0	0	0	70	8.1	10	22	1.25	

had dropped to 34 mg., non-protein nitrogen to 70 mg. and creatinine to 3 mg. On the 19th day the animal was functionally normal. The experiment was terminated.

The kidneys appeared normal in both color and size. The capsule was not adherent, and there was a normal relationship in volume between cortex and medulla. The glomeruli were not prominent and there was no evidence of congestion. The microscopic study showed many of the glomeruli to be apparently increased in size. The capillary walls were prominent and the nuclei of the endothelium hyperchromatic and hyperplastic. Between the capillary loops were connective tissue nuclei. In some areas the loops had become obliterated, in others they were distended with blood. The epithelium of the proximal convoluted tubules has shown an advanced stage of repair in terms of regenerating epithelial cells, not of an atypical character, but cells normal for this location in the tubule. Mitotic figures were frequently present in these regenerating specialized cells. In other cells in which the process of repair had been completed the cytoplasm stained uniformly and the nuclei were hyperchromatic (Fig. 2).

This animal has passed from a state of marked renal functional depression except for the volume output of urine, to one of normal renal function. The renal pathology associated with the former state consisted in edema and early changes of necrosis of the convoluted tubules with a prominence of the capillary endothelium and a distention of the capillaries with blood. The renal pathology associated with the latter functional state consisted in a repair to the epithelial injury and the development of early connective tissue changes in the glomeruli.

The course of the nephritis in the animal of Experiment 17, Table II, Group II, is characteristic of those animals of the group in which the recovery process was much more gradual, in this instance extending over a period of 7 months.

On the 2nd day following the subcutaneous injection of uranium the animal became polyuric with an output of 783 cc. of urine which contained 2 gm. of albumin per liter, glucose and granular casts. The elimination of phenolsulphonephthalein was reduced from 75 per cent to 10 per cent and the reserve alkali from 8.1 to 7.95. There was no appreciable retention of urea, non-protein nitrogen or creatinine. On the 7th day of the nephritis there was 537 cc. of urine which contained 0.5 gm. of albumin, numerous granular casts and a few hyaline casts. The elimination of the dye had been reduced to 5 per cent and the reserve alkali to 7.8. There was a retention of blood urea of 70 mg., non-protein nitrogen of 120 mg. and creatinine 4.75 mg. On this day the animal was anesthetized and a wedge-shaped piece of tissue removed from the left kidney. This tissue showed the usual changes characteristic of the acute stage of the nephritis from uranium. On the 24th day of the nephritis the animal formed 400 cc. of urine which was free from albumin but contained finely granular and hyaline casts. The elimination of phenolsulphonephthalein had increased from 5 to 45 per cent and the reserve alkali had increased to 8.1. The retention of blood urea was reduced from 70 to 15 mg., non-protein nitrogen from 120 to 30 mg. and creatinine from 4.75 to 1.5

TABLE II.

Group II. Experiment 17. Duration of Experiment, 9 Months and 15 Days.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphonephthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations 9/17/26	10.2 kg. 1–2 yrs.	0	500	375	0	0	0	0	75	8.1	15	32	1.1	Kidneys appear normal. Capsule not thickened but adherent in areas to cortex. On section tissue cuts without resistance. No evidence of congestion. Cortex not decreased in volume
9/24/26		4	500											
9/26/26 2nd day of intoxication		0	500	783	2	0.16	0	Numerous granular	10	7.95	16	35	1.1	
10/1/26 7th day of intoxication	Kidney tissue removed	0	500	537	0.5	0.15	Trace	Numerous granular. Few hyaline	5	7.8	70	120	4.75	Microscopic study shows glomeruli to vary much in size and structure. Many units show connective tissue obliteration of loops, while other loops contain blood. Capsules definitely thickened by connective tissue and in areas capillary loops adherent to fibrous capsules. Formation of intertubular connective tissue
10/12/26 18th day of intoxication		0	500	590	Trace	Trace	0	Few granular. Increase in hyaline	45	7.95	50	75	3.75	
10/18/26 24th day of intoxication		0	500	400	0	0	0	Few finely granular. Few hyaline	45	8.1	15	30	1.5	Epithelium of convoluted tubules shows 2 entirely different types of repair. 1st type similar to that of Experiment 27, Table I, Group II,

3/12/27 5 mos. and 18 days after uranium		0	500	473	0	0	0	No granular casts. Few hyaline	65	8.1	15	32	1.5	in which normal convoluted tubule cells have been replaced by process of mitosis. 2nd type of tubular repair accomplished by formation of
4/30/27 7 mos. and 6 days after uranium		0	500	525	0	0	0	0	75	8.1	15	30	1.2	flattened, non-specialized cytoplasmic layer on basement membrane of convoluted tubules. In such regenerated tubules cell differentia-
7/9/27 9 mos. and 15 days after uranium		0	500	500	0	0	0	0	75	8.1	10	26	1.2	tion frequently imperfect. Nuclei stain intensely and are prominent

mg. This type of functional response, a urine with no albumin or a trace, a few hyaline and finely granular casts, a low phenolsulphonephthalein excretion but with no retention, continued until the 7th month following the commencement of the nephritis, when the functional response of the animal became normal.

At the autopsy the kidneys appeared normal in size. The capsule was adherent in small areas. On section there was no evidence of congestion and there was no disproportion in volume between the cortex and the medulla. The microscopic study showed the glomeruli to vary much in size and in structure. Many of these units showed connective tissue obliteration of the loops while other capillaries contained blood. The glomerular damage was not uniform. The capsules showed a variable degree of thickening and frequently the capillary tufts were in part adherent to the capsules. The epithelium of the convoluted tubules showed two entirely different types of repair. The first type is similar to that of Experiment 27, Table I, Group II, in which normal convoluted tubule cells have been replaced by a process of mitosis from such cells which have not undergone too severe epithelial injury. The second form of tubular repair is by an entirely different type of cell. This type of repair takes place in those tubules in which the severest epithelial destruction has occurred and develops as an ingrowth from the lower end of the proximal convoluted tubule or from the upper end of the descending limb of Henle's loop of a flattened, at first non-specialized cytoplasmic layer in which cell differentiation is frequently imperfect. Later as cell differentiation develops, these cells acquire more cytoplasm, stain uniformly and have conspicuous, deeply staining nuclei. The functional significance of these two types of cells involved in the epithelial repair of the convoluted tubules will be discussed in a second study. Around many of the glomeruli, between the tubules and in areas once occupied by tubules, there is a connective tissue formation both in its cellular and fibrous stages (Fig. 3). This animal, as was the case with the animal of Experiment 27, passed from a state of definite renal functional depression to one of normal renal function. The length of time necessary for the animal of Experiment 17 to make such a functional transition was much longer. The histological changes developing in the kidney associated with a restoration of function were in general of the same character in the two animals and consisted in epithelial repair to the convoluted tubule epithelium and obliterative, fibrous tissue changes in the glomeruli.

Conclusions for Group II.

1. The animals used in this group developed, as did the older dogs of Group I, a severe acute nephritis. The fact that such an acute nephritic process existed in these animals is not only established by a comparison of their functional response with the animals of Group I, but by obtaining tissue from the kidneys of four of the animals during the acute nephritis and before any evidence of a return towards the normal in function had developed.

2. The functional response shown by the animals of Group II was similar in character but not so severe as that shown by the animals of Group I. The animals of Group II became polyuric with an albuminous urine containing casts. There was a rapid reduction in the elimination of phenolsulphonephthalein, a decrease in the reserve alkali and a retention of urea, non-protein and creatinine. At such a stage of impaired renal function the kidneys showed a marked injury to the convoluted tubule epithelium and glomerular changes which consisted of an acute congestion of the capillary loops. The endothelial cells of the walls of the capillaries are prominent and their nuclei stain intensely. Associated with the epithelial repair to the convoluted tubules and coincident with the development of progressive fibrous tissue changes in the glomeruli, there occurred a decrease and final absence of albumin from the urine and the character of the casts changed from a granular to a hyaline type and disappeared. There has developed a gradual increase in the elimination of phenolsulphonephthalein, a decrease in the reserve alkali of the blood and a decrease in the retention of urea, non-protein nitrogen and creatinine, until these values have reached the normal functional values for the animals. Likely depending upon the severity of the epithelial injury and the type and completeness of the epithelial repair which followed, the rapidity with which the animals reestablish a functional normal varied in different members of the group.

Group III. Animals that, following the Development of an Acute Nephritis, Failed to Return to a Functional Normal. Animals with a Chronic Uranium Nephritis.

Of the seventy-two dogs which were used in the present study, thirty-six of the number developed such evidence of altered renal function and have shown such chronic changes in the kidneys that they can be classed as animals with a chronic nephritis. The duration of such processes existed in the animals from 2 years and 3 months to 5 years and 4 months. In six of these animals during the stage of acute nephritis a wedge-shaped piece of kidney tissue was removed to serve as an anatomical control for the existing acute functional disturbance and for subsequent changes in the kidneys which might develop from the nephritis. The experiments in this group were terminated at vari-

TABLE I.

Group III. Experiment 21. Duration of Experiment, 2 Years and 8 Months.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphone-phthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations 10/7/25	16.2 kg. 1–2 yrs.	0	500	400	0	0	0	0	90	8.05	15	28	1.3	Acute renal injury observed in tissue removed on 13th day of experiment showed kidney acutely swollen and congested. Glomeruli prominent and capillaries distended with blood. Granular material without fibrin fibrils is occasionally found in subcapsular spaces. Endothelium of capillaries appears swollen with increase of deeply staining nuclei. Convoluted tubule cells edematous and frequently necrotic. In 2 tubules occasional mitotic figure seen. No actual repair of epithelium. Cells of Henle's loops appear normal
10/7/25		4	500											
10/10/25 3rd day of intoxication		0	500	870	3.	1.3	Present	Numerous granular	15	7.9	25	42	1.3	
10/15/25 8th day of intoxication		0	500	942	2.1	0.8	Present	No change	0	7.8	49	93	4.3	
10/20/25 13th day of intoxication	Kidney tissue removed	0	500	978	2.8	Trace	Present	No change	5	7.8	62	131	6.1	
11/10/25 24th day of intoxication		0	500	533	0.8	0	0	Numerous granular. Few hyaline	15	7.9	41	91	4.0	Terminal pathology of kidney in general similar to that of animal of

2/10/26 4 mos. after uranium		0	500	730	Present	0	0	Few finely granular. Numerous hyaline	48	8.05	26	58	2.1	Experiment 66, Group III. Glomerular changes more advanced. Connective tissue obliteration of glomerular capillaries more uniform and more marked. Hyalinization of capillary tufts frequently observed
11/10/26 13 mos. after uranium		0	500	685	Present	0	0	No change	36	8.05	31	62	2.0	
9/10/27 1 yr. and 11 mos. after uranium		0	500	874	Present	0	0	Few hyaline casts	46	8.05	24	58	1.7	
12/20/27 2 yrs. and 2 mos. after uranium		0	500	730	Present	0	0	No change	31	8.1	20	44	1.8	
4/20/28 2 yrs. and 6 mos. after uranium		0	500	815	Present	0	0	No change	48	8.05	18	47	2.0	
6/20/28 2 yrs. and 8 mos. after uranium		0	500	983	Present	0	0	No change	35	8.05	23	52	1.8	

ous stages in the nephritis as might be indicated by changes in function, and finally in a larger number of animals after sufficient time was allowed as determined by changes in the blood and urine to indicate that the animals were unable to effect any further functional improvement. The animals of Experiments 66 and 21 are representative of the course of the chronic nephritis in this group of thirty-six dogs.

On the 6th day of the acute nephritis in the dog of Experiment 66 there was an output of 1200 cc. of urine which contained 2 gm. of albumin per liter, glucose and acetone with numerous granular and hyaline casts. There was no elimination of phenolsulphonephthalein in a 2 hour period, the reserve alkali was reduced from the normal of 8.1 to 7.8; there was a retention of blood urea of 45 mg., non-protein nitrogen of 78 mg. and 4.6 mg. of creatinine. At this period of the experiment renal tissue was removed as a control. The changes in this tissue were similar to those previously described for the animals of Group I and Group II at the height of the acute nephritis. On the 25th day of the nephritis the amount of albumin had decreased along with a decrease in the volume output of urine with but apparently little change in the number of casts. There was a trace of phenolsulphonephthalein eliminated in a 2 hour period and the reserve alkali of the blood had increased to 7.9. The evidence of retention had increased so that the blood showed 100 mg. of urea, 128 mg. of non-protein nitrogen and 6.1 mg. of creatinine. On the 35th day of the experiment there was an output of 511 cc. of urine which contained albumin but with a marked decrease in granular casts and an increase in the hyaline type. The elimination of phenolsulphonephthalein was 12 per cent and the reserve alkali of the blood had increased to 7.95. The retention of urea nitrogen had decreased to 33 mg., non-protein nitrogen to 50 mg. and creatinine to 3.1 mg. From this period to the termination of the experiment, 2 years and 3 months from its commencement, the functional response of the animal varied but little. At this time the 24 hour output of urine was variable. On the last day of the experiment there was an output of 811 cc. of urine which contained albumin and hyaline casts. There was an elimination of 34 per cent of phenolsulphonephthalein, a normal alkali reserve of the blood of 8.1, a retention of blood urea of 30 mg., non-protein nitrogen of 54 mg. and creatinine 2.4 mg. The results obtained in the second type experiment for the group, Experiment 21, Table I, Group III, are similar to those described for the preceding animal. In the animal of Experiment 21 renal tissue was removed on the 13th day of the acute nephritis to use for control purposes. The experiment was terminated 2 years and 8 months after the commencement of the acute nephritis.

The pathology of the kidney during the acute stage of the injury was similar in type to that described for the animals of Groups I and II (Fig. 4). The chronic renal pathology developing as a result of an attempt at repair with a failure to reestablish normal function was as follows. The kidneys are small and the surface irregular. The capsule is thickened and adherent. The tissue cuts with resistance

and on section there is a marked thinning of the cortex. The histological study showed a general glomerular fibrosis which varied in intensity in different glomeruli. Many of the capillary loops are obliterated by connective tissue formation. In a few of the glomeruli this process has been complete with a later hyalinization of the fibrous tissue. In other units the obliteration has been partial with the patent loops distended with blood. The glomerular capsules showed a connective tissue thickening to which the fibrous capillaries may be adherent. Hyperplasia of the capsular epithelium has been rarely observed. Many of the tubules have disappeared and their place has been taken by masses of fibrous tissue. The smaller arteries are numerically prominent. In such vessels there is a definite thickening of the middle coat. The proximal convoluted tubules are lined by two types of cells. The first type in some of which processes of degeneration may still be noted have in general the appearance of convoluted tubule epithelium. Such cells which are in the process of degeneration appear granular, stain imperfectly and show evidence of nuclear disintegration. Other cells of this type have shown evidence of repair by the presence of mitotic figures. The second type of cell in the regenerated epithelium of the convoluted tubules is atypical both in appearance and in location. The cells when differentiated are flattened, stain uniformly and have prominent nuclei. In other tubules this regenerated epithelium appears as a syncytial layer with imperfectly differentiated cells. Casts were frequently present in such tubules, the loops of Henle and in the collected tubules (Figs. 5 and 6).

Conclusions for Group III.

1. A chronic nephritis was produced in the dog which involves all of the histological elements of the kidney by the use of one subcutaneous injection of 4 mg. of uranium nitrate per kilo. In such animals the sequence of the histological changes in the kidneys in correlation with their functional expression has been studied in dogs from the commencement of the injury until the chronic nehritis has existed for 5 years and 2 months.

2. As early as the 2nd day following the development of an acute nephritis, the animals became polyuric with an albuminous urine containing granular casts. There was a marked reduction in the elimination of phenolsulphonephthalein, or it failed to appear in the urine in a 2 hour period. The reserve alkali of the blood was reduced to as low as 7.8 and a retention occurred in urea nitrogen, non-protein nitrogen and creatinine. At such periods of renal functional depression there was slight evidence of structural glomerular injury but marked evidence of proximal convoluted tubule degeneration and necrosis.

The severity of these functional changes increased to a period in the respective experiments which varied between the 6th and the 16th days. At such a period the amount of albumin and number of granular casts in the urine diminished, phenolsulphonephthalein reappeared in the urine or increased in its percentage elimination; the reserve alkali of the blood increased and the retention of urea, non-protein nitrogen and creatinine decreased. Such functional expressions of improvement were coincident with the commencement of epithelial regeneration in the proximal convoluted tubules and structural changes of a chronic degenerative character in the glomeruli. The animals of this group, unlike those of Group II, failed to reestablish a functional normal. The explanation for this difference has not been ascertained but is likely dependent upon the interrelationship which exists between the maintenance of epithelial repair, the degree of obliterative changes in the smaller renal vessels and the progressive fibrosis and hyalinization of the glomeruli. In the animals of Group II there was more histological evidence of epithelial repair and less evidence of vascular and glomerular injury.

In conclusion, the experiments permit certain general deductions:

1. Uranium nitrate in the dog induces an epithelial injury to the kidney which is very largely localized to the proximal convoluted tubule epithelium. With the development of such structural changes in this tissue, functional alterations are induced in the glomeruli which later in the experiments are shown structurally by the formation of connective tissue in these units. Other than the water elimination by the kidney, the other evidence of impaired renal function progressively increases as the epithelial injury becomes intensified.

2. A certain number of dogs rendered acutely nephritic from uranium, after a variable period allowed for renal repair, returned to a complete functional normal. The commencement of such a restoration in function is coincident with the development of the process of epithelial repair in the injured tubular epithelium and at the same time with the development of histological evidence of structural changes in the glomeruli. The ability of such animals to effect a restoration in normal renal function depended upon the completeness and type of epithelial repair which develops and not with the degree to which connective tissue formation and obliteration of glomeruli has failed to develop.

3. A final group of animals, after having developed an acute nephritis, were unable to effect such changes of repair in the kidneys that a normal functional state could be established. These animals showed the anatomical characteristics and the functional expression of a chronic nephritis. Such a failure to return to a state of functional normal has been associated in them with the regeneration in the proximal convoluted tubules of a predominant type of epithelium which is atypical for the tubules in this location and with the development of structural changes in the smaller arteries of the kidney and obliterative changes in the glomeruli.

4. The study as a whole emphasizes the functional value of the proximal convoluted tubule epithelium during periods of acute renal functional depression, when such periods are recuperated from with the establishment of a state of normal renal function, and in conditions in which without a complete restoration of function a functional improvement has developed with the establishment of a chronic nephritis.

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EXPLANATION OF PLATES.

PLATE 31.

FIG. 1. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 50, Table I, Group I. The experiment was terminated at the height, as indicated by functional changes, of an acute nephritis from uranium. At *a*, is shown an apparently normal glomerulus with capillary loops distended with blood. The capillary endothelium is prominent with deeply staining nuclei. At *b*, are shown proximal convoluted tubules in various stages of edema, vacuolation and necrosis. The cells in the ascending and descending limits of Henle's loops at *c*, appear normal. Such tubules contain casts.

FIG. 2. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 27, Table I, Group II, and represents the changes which have developed following the acute nephritis with a return to a state of normal renal function. The glomerulus at *a*, appears larger than normal. The capillary walls are prominent and the nuclei are hyperchromatic and hyperplastic. Connective tissue nuclei are found between the capillary loops. In some areas the loops have become obliterated. The epithelium of the convoluted tubules at *b*, show an advanced stage of repair by the formation of an epithelium characteristic for this part of the tubule. Mitotic figures are frequently present in such regenerating, specialized cells. The atypical type of flattened epithelium has not participated in the process of epithelial repair.

PLATE 32.

FIG. 3. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 17, Table II, Group II. The figure represents another type of repair process which has developed in animals of the group and which has resulted in a restoration of normal renal function following the period of depressed function which existed during the stage of the acute nephritis. At *a*, is shown a glomerulus with a fibrous capsule. The glomerular capillaries show an increase in nuclei, many of the loops have been obliterated while others contain blood. The epithelial repair has been effected by two types of cells. At *b*, are shown regenerating epithelial cells of the type characteristic for the convoluted tubules. The cells contain mitotic figures; At *c*, is shown a relining of these tubules by a flattened, atypical type of cell. Periglomerular and intertubular connective tissue is prominent.

FIG. 4. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 21, Table I, Group III. From tissue removed on the 13th day of the acute nephritis before any evidence of an improvement in renal function had developed. The figure shows at *a*, a glomerulus with capillaries distended with blood. The capillary walls are prominent and the endothelial nuclei hyperchromatic. There is no histological evidence of acute degenerative changes in the glomeruli. At *b*, are shown proximal convoluted tubules in various stages of edema and necrosis. There is no evidence of epithelial repair. At *c*, is shown the epithelium in the descending limb of Henle's loop which appears normal. There is no edema or necrosis. The tubule contains a cast.

PLATE 33.

FIG. 5. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the same animal of Experiment 21, 2 years and 8 months after the acute nephritis when the animal had developed both functional and anatomical evidence of a chronic nephritis. The figure shows at *a*, glomeruli in an advanced stage of connective tissue formation. Many of the capillary loops have been obliterated and the capsules are thickened. At *b*, is shown a replacement of the normal convoluted tubule epithelium by an atypical type of flattened cell which is frequently imperfectly differentiated. The smaller renal arteries are shown at *c*, with marked thickening of the middle coat. There is an overgrowth of intertubular and periglomerular connective tissue at *d*.

FIG. 6. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is also from the animal of Experiment 21. At *a*, are shown parts of two glomeruli with a marked thickening of the capsule, connective tissue obliteration of the capillary loops and periglomerular connective tissue. The epithelial regeneration of the proximal convoluted tubules has taken place by two types of cells. At *b*, is shown the regeneration by a type of cell normal for the convoluted tubules. At *c*, these tubules are shown to be lined with a flattened, frequently undifferentiated cytoplasmic layer. Between the tubules and taking the place of those which have failed to effect a repair as shown at *d*, is connective tissue in its cellular and fibrous stages of formation.

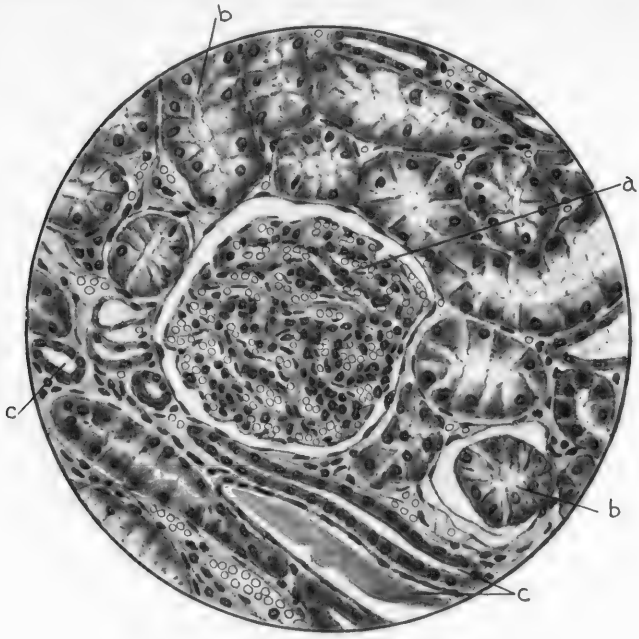

FIG. 1.

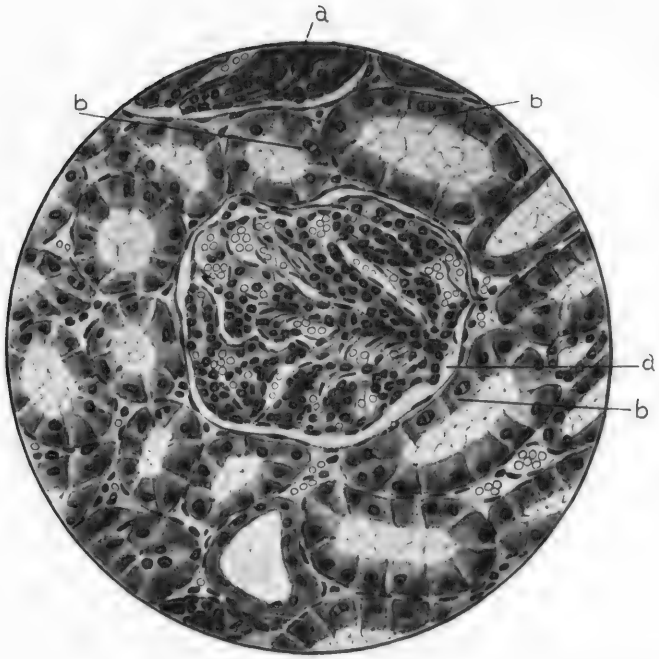

FIG. 2

(MacNider: Chronic nephritis by uranium nitrate.)

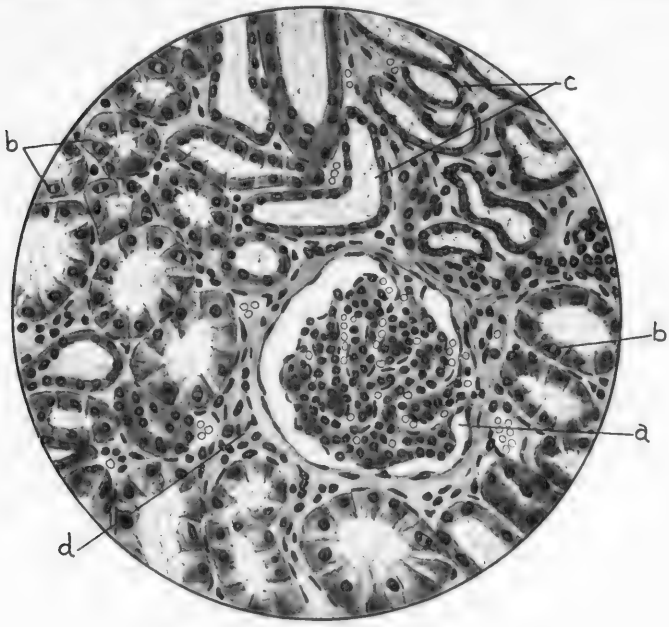

FIG. 3.

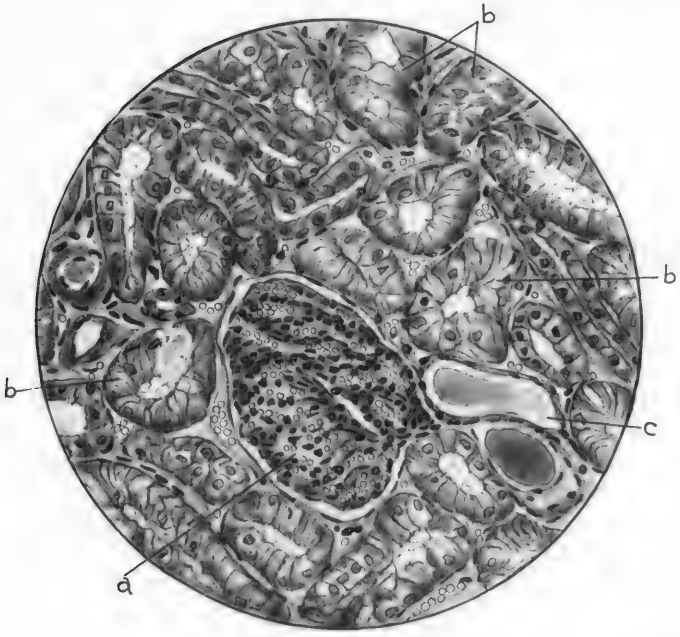

FIG. 4.

(MacNider: Chronic nephritis by uranium nitrate.)

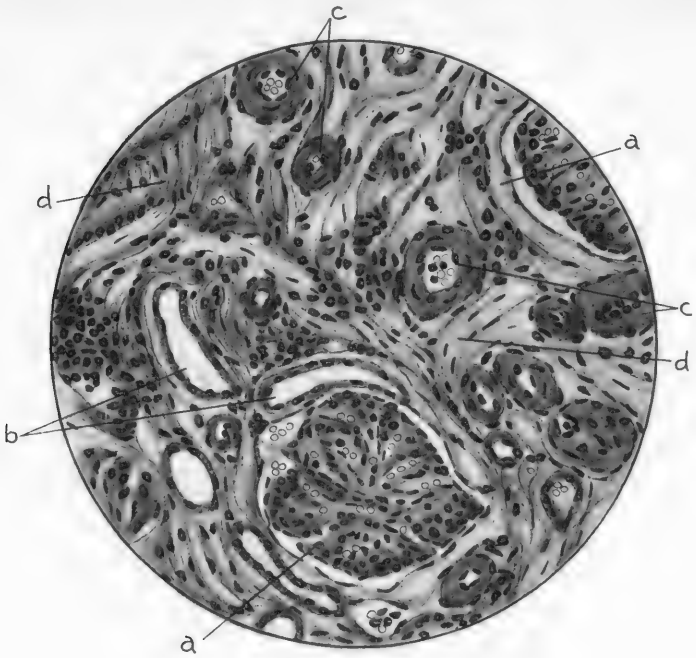

FIG. 5.

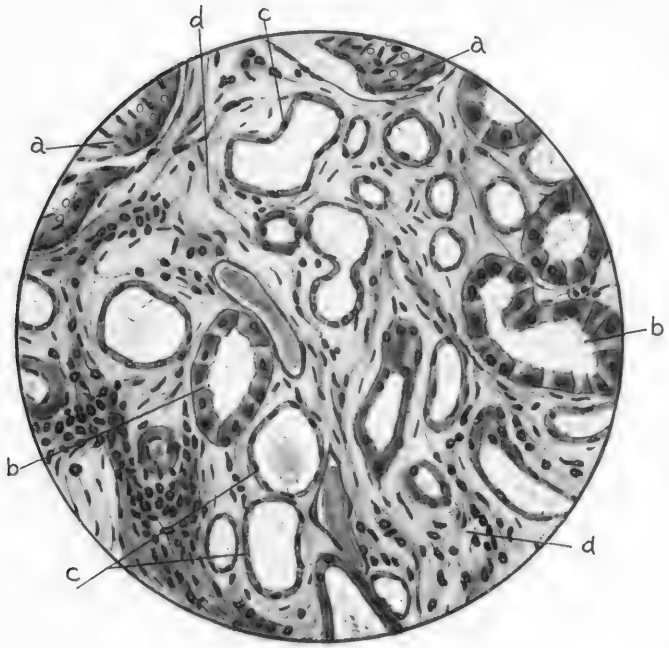

FIG. 6.

(MacNider: Chronic nephritis by uranium nitrate.)

THE FUNCTIONAL AND PATHOLOGICAL RESPONSE OF THE KIDNEY IN DOGS SUBJECTED TO A SECOND SUBCUTANEOUS INJECTION OF URANIUM NITRATE.*

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PLATES 34 TO 37.

(Received for publication, November 3, 1928.)

In the preceding study it was shown that a certain number of dogs (Group II) rendered acutely nephritic from uranium, after having developed the functional response characteristic of such an injury returned to a complete functional normal. It was, furthermore, shown by a study of this group, that their ability to make such a functional readjustment depended upon the power of the respective animals to regenerate or replace in the proximal convoluted tubules two entirely different types of epithelium. In certain of the animals the regenerated epithelium resembled histologically normal proximal convoluted tubule cells, while in other animals in which a similar return in functional response was obtained, there was in addition to the regeneration of the above type of cell, a dominance in regeneration of a low, flattened, apparently less specialized epithelium. The object of the present study is to ascertain by subsequent injections of uranium if there is any difference in the susceptibility of these types of regenerated epithelium to this nephrotoxic agent, and if so, what variation can be obtained in the functional response of such secondarily injured kidneys.

In 1912 Suzuki (1) during his studies on the localization of the nephrotoxic action of uranium described the regeneration of convoluted tubule epithelium and noted a resistance which such animals had developed to a later intoxication by it.

* Aided by a grant from the Ella Sachs Plotz Foundation.

Later than this, Aschoff (2), in reviewing Suzuki's work discussed the increased resistance of the renal epithelium to uranium and interpreted it as a defense mechanism. He did not specifically describe the morphology of the epithelium which had become resistant. In 1916 (3) a study was conducted in this laboratory on animals with a naturally acquired chronic nephritis in which it was shown, first, that a specific type of regenerated epithelium in the convoluted tubules was resistant to an injury from uranium, second, that normal convoluted tubule epithelium failed to manifest this resistance, and, third, that the regenerated cells were furthermore resistant to the toxic action of a general anesthetic. In 1924 Gil y Gil (4), in his research on the immunity of kidney cells to uranium, considered it possible to immunize the kidney of the rabbit against uranium, and that such a state was accomplished by the partial destruction of renal tissue which lead to a parenchymatous contracted kidney. He was able to show by biological methods, as Karsner, Reiman and Brooks (5) had previously shown by chemical methods, that the parenchymatous injury was coincident with the elimination of uranium by the kidney. Very recently Hunter (6) has described the acute renal injury in the rabbit kidney from uranium, has noted the changes of repair and has described the resistance or immunity of the epithelium to uranium.

In the following study the cytological basis for the resistance of the kidney of the dog to uranium will be discussed and the functional basis of this resistance, both when it occurs and when it fails to occur, will be considered in connection with the pathology of the kidney.

Method.

Forty-one dogs varying in age from 1 to 2 years were used in the experiments. The technique employed, with certain exceptions which will be noted, was identical with that followed in the preceding paper. In the present series of animals, after having obtained the normal observations, the dogs were given 2 mg. of uranium nitrate per kilo subcutaneously. Later in the experiments this amount of uranium, or twice the amount, was given as a secondary injection. In such experiments it becomes necessary to establish as controls, first, that the above amount of uranium will not only induce an acute nephritis, but such a reaction of repair on the part of the animals that a chronic nephritis will develop, second, to ascertain the effect of a second injection of uranium in an animal with an acute nephritis when functionally there is little evidence of recuperation, and, third, after a repair with recuperation has been effected to ascertain what is the anatomical difference in such states of recuperation which may determine the susceptibility or lack of susceptibility of the kidney to uranium.

Eight of the forty-one dogs developed an acute nephritis of such severity that they died from it or the experiments were terminated during its course. Such animals failed to develop any functional improvement and at autopsy showed the usual changes characteristic of this stage of the injury. Four of the remaining

thirty-three animals of the series were not given a second injection of uranium but were permitted to effect a renal repair with the development of a chronic nephritis. The course of the nephritis in the control animal of Experiment 8, Table I, is characteristic of this group and is in general similar to the animals of Group III in the previous study. The findings as indicated by the table do not require any further elaboration.

The Functional and Pathological Response of the Kidney in Dogs Subjected to a Second Subcutaneous Injection of Uranium Nitrate.

Secondary injections of uranium were made in dogs at three different stages of their reaction from the initial injection. During the course of the acute nephritis when there was either slight or no evidence of an improvement in renal function, after the animals had returned to a functional normal or after they had developed a chronic nephritis. In the last two groups in order to ascertain anatomically the existence of a chronic nephritis, renal tissue was removed from the upper pole of the left kidney to serve as a control before the secondary injection of uranium was made.

Eight animals were given a secondary injection of 2 mg. of uranium during the course of the acute nephritis when there was either no evidence of an improvement of renal function or when this evidence was slight. The control animal of Experiment 11, Table II, indicates the course of the nephritis in such animals.

Following the first injection of uranium the animal developed an acute nephritis which reached its height functionally on the 9th day. The output of urine, which was 749 cc., contained 2.1 gm. of albumin per liter and numerous granular casts. The elimination of phenolsulphonephthalein was reduced from the normal of 77 per cent to 6 per cent, and the reserve alkali of the blood from 8.1 to 7.85. There was a retention of blood urea of 56 mg., non-protein nitrogen 124 mg. and creatinine 2.9 mg. On the 16th day of the acute nephritis after the animal had shown a slight functional improvement, the second injection of 2 mg. of uranium per kilo was made. The dog had lost only 0.5 of a kilo in body weight. Following the second injection there was a rapid increase in the indications of diminished renal function. On the 6th day following the injection the output of urine was reduced to 73 cc. and the amount of albumin had increased to 3.4 gm. There was no elimination of phenolsulphonephthalein, the reserve alkali was reduced to 7.7 and there was a retention of urea nitrogen of 123 mg., non-protein nitrogen 289 mg. and creatinine 7.8 mg. On the 7th day the animal died in convulsions.

The pathological study of the kidneys showed them diffusely congested. The capsule was not adherent and when incised the swollen cortex appeared in the line

TABLE I.

Control Experiment 8. Duration of Experiment, 4 Months and 10 Days.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphonephthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observation 10/15/27	14.7 kg. 1–2 yrs.	0	500	484	0	0	0	0	86	8.05	14	38	1.2	Kidneys appear normal. Capsule not thickened. Glomeruli show early capsular and intercapillary fibrosis. Definite increase in periglomerular and intertubular connective tissue
10/17/27		2	500											
10/19/27. 2nd day of intoxication		0	500	741	1.8	Present	Present	Numerous granular	21	7.9	35	71	1.2	Epithelium lining convoluted tubules shows evidence in different tubules of degeneration and repair. Degenerative changes consist in cloudy swelling, edema and rarely necrosis. Processes of repair are shown in replacement of normal tubular epithelium by flattened, frequently undifferentiated cytoplasmic layer with prominent nuclei
10/25/27. 8th day of intoxication		0	500	918	2.0	Present	Present	No change	8	7.85	52	131	3.4	
11/17/27. 1 mo. after intoxication		0	500	514	Present	0	0	Few finely granular. Numerous granular	14	7.95	35	78	3.0	
2/27/28. 4 mos. and 10 days after intoxication		0	500	607	Present	0	0	No change	48	8.0	28	51	2.0	

of incision. The cortex was pale in contrast with the medulla, and the glomeruli were prominent. The capillaries were distended with blood, and there was occasionally observed a granular exudate containing fibrin in the subcapsular spaces. There was no periglomerular connective tissue hyperplasia. The proximal convoluted tubule epithelium was in general severely swollen, edematous and necrotic. There was no evidence of epithelial repair either by the regeneration of epithelium normal for the convoluted tubules or by the formation of the flattened type of epithelial cell.

The animals of these six experiments showed a complete lack of resistance to a second injection of uranium when the nephrotoxic agent was given before processes of repair had taken place in the kidney with a return of its function definitely towards the normal. The second injection of uranium served to increase the severity of the epithelial degeneration and intensify the evidence of functional impairment (Fig. 1).

In the second group of ten animals the secondary injection of uranium was made after the dogs had established a complete functional recovery from the first injection. Prior to the second injection kidney tissue was removed to serve as a control for the renal changes developing from the second use of uranium. The control animal of Experiment 21, Table III, is representative of this group.

On the 6th day of the acute nephritis the output of urine was 842 cc. It contained 1 gm. of albumin per liter with granular casts. The elimination of phenolsulphonephthalein had been reduced from the normal of 79 per cent to 8 per cent and the reserve alkali from 8.1 to 7.85. There was a retention of 61 mg. of urea, 128 mg. of non-protein nitrogen and 3.2 mg. of creatinine. On the 24th day, judging from the functional findings, the animal had returned to the normal. There was an output of 470 cc. of urine which did not contain albumin and which was free from casts. The elimination of phenolsulphonephthalein was 68 per cent, the reserve alkali of the blood was normal and there was no evidence of retention. A wedge-shaped piece of tissue was removed from the upper pole of the left kidney. The tissue showed the glomerular capillaries distended with blood and an endothelium which appeared swollen with prominent nuclei. There was no exudate in the subcapsular spaces and no intracapillary or periglomerular connective tissue formation. The epithelium of the convoluted tubules showed marked evidence of regeneration by the formation of cells pyramidal in shape, containing deeply staining nuclei or nuclei in the process of mitotic division. The cells were of the type normal for the proximal convoluted tubules. In other tubule cells these changes had not progressed to the same degree of repair and the epithelium showed evidence of degeneration. The epithelial regeneration in this animal had not been

TABLE II.

Control Experiment 11. Duration of Experiment, 23 Days.

Data from the first intoxication by uranium nitrate.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphonephthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood.	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations 10/20/27	12.2 kg. 1–2 yrs.	0	500	312	0	0	0	0	77	8.1	15	36	1.1	Kidneys show a diffuse congestion. Capsule not adherent. When capsule is incised swollen cortex bulges into incision. On section cortex is pale as contrasted with medulla. Glomeruli prominent. Glomerular capillaries distended with blood. Endothelium swollen and nuclei prominent. Capsules not thickened. No periglomerular connective tissue hyperplasia
10/22/27		2	500											
10/24/27. 2nd day of intoxication		0	500	608	1.3	0.4	Present	Numerous granular	31	7.9	41	80	1.1	
11/3/27. 9 days after intoxication		0	500	749	2.1	Present	Present	Numerous granular	6	7.85	56	124	2.9	
11/8/27.14th day of intoxication		0	500	547	0.2	0	0	Numerous granular and hyaline	18	7.95	38	81	2.5	
11/10/27. 16th day of intoxication	11.7 kg.	2	500	Data from the second intoxication by uranium nitrate.										Convoluted tubule epithelium in general severely swollen and vacuolated with areas of necrosis. In a study of large number of sections only 3 mitotic figures have been seen. Flattened type of regenerated epithelium has not appeared in tubules. No definite evidence of repair
11/12/27. 2nd day of 2nd intoxication		0	500	342	1.4	Trace	Present	Numerous granular and few hyaline	5	7.8	76	159	3.8	
11/14/27. 4th day of 2nd intoxication		0	500	111	4.2	Trace	Trace	No change	Trace	7.7	114	271	7.2	

11/16/27. 6th day of 2nd intoxication		0	500	73	3.4	0	Trace	No change	0	7.7	123	289	7.8	Epithelium of loops of Henle appears normal
11/17/27. 7th day of 2nd intoxication	Convulsions. Died in air hunger													Experiment shows no resistance to 2nd uranium injection when given before processes of repair have taken place in kidney with return of its function toward normal. 2nd injection of uranium increases the epithelial degeneration

TABLE III.

Control Experiment 21. Duration of Experiment, 33 Days.

Data from the first intoxication by uranium nitrate.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphonephthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations 10/15/27	16.4 kg. 1–2 yrs.	0	500	471	0	0	0	0	79	8.1	12	34	1.3	Microscopic study of tissue removed on 24th day shows many glomeruli which appear larger than normal. Glomerular endothelium contains prominent nuclei. Capillaries engorged with blood. No subcapsular exudate. Capsules not thickened, no periglomerular fibrosis Convoluted tubule epithelium shows 2 types of changes. Evidence of repair seen in occurrence of mitotic figures in epithelium which in general is characteristic for cells in this part of tubule. Other areas show these cells in various stages of injury as indicated by cloudy swelling and edema
10/17/27		2	500											
10/19/27. 2nd day of intoxication		0	500	887	2.1	1.0	Present	Numerous finely and coarsely granular	38	7.95	33	64	1.4	
10/21/27. 4th day of intoxication		0	500	1085	2.3	Present	Present	Numerous coarsely granular	15	7.95	48	90	2.4	
10/23/27. 6th day of intoxication		0	500	842	1.0	Trace	Present	No change	8	7.85	61	128	3.2	
10/29/27. 12th day of intoxication		0	500	561	Present	0	0	Numerous finely granular. Few coarsely granular	21	7.95	40	78	2.2	

11/2/27. 16th day of intoxication		0	500	681	Present	0	0	No change	48	8.0	20	38	1.8
11/10/27. 24th day of intoxication	Kidney tissue removed	0	500	470	0	0	0	0	68	8.1	14	32	1.2
11/12/27. 26th day of intoxication	14.8 kg.	2	500	Data from the second intoxication by uranium nitrate.									
11/14/27. 2nd day of 2nd intoxication		0	500	879	2.0	1.1	Present	Numerous coarsely and finely granular	20	7.9	42	91	2.3
11/16/27. 4th day of 2nd intoxication		0	500	381	2.3	0.8	Present	No change	Trace	7.8	86	172	4.8
11/19/27. 7th day of 2nd intoxication	Semi-comatose. Air hunger. Killed.	0	500 vomited.	105	1.1	Trace	Present	No change	0	7.7	105	214	6.8

No epithelial replacement by flattened, less specialized epithelial lining of convoluted tubules which is resistant to uranium

At termination of experiment kidneys were swollen and cortex pale with prominent glomeruli. Histologically many glomeruli appeared normal except for intense congestion of capillaries and prominence of endothelium. In others there was matting together of the loops, increase in nuclei and thickening of capsule. Changes in convoluted tubules were those of edema, vacuolation and severe necrosis. No evidence of repair. Epithelium of descending limb of Henle's loop appeared normal

Epithelial repair of kidney by type of specialized epithelium resembling convoluted tubule epithelium has no resistance to 2nd injection of uranium

TABLE IV.

Experiment 3. Duration of Experiment, 3 Months and 18 Days.

Data from the first intoxication by uranium nitrate.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphonephthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations 10/18/27	18.7 kg. 1–2 yrs.	0	500	482	0	0	0	0	74	8.0	15	37	1.4	Changes in kidney have resulted from 2nd injection of 2 mg. of uranium nitrate per kg.
10/23/27		2	500											
10/25/27. 2nd day of intoxication		0	500	748	2.3	Present	Present	Numerous granular	15	7.85	32	57	3.0	Glomerular pathology is similar to that described for animal of Experiment 8, Table I. Definite periglomerular and intertubular formation of connective tissue; both cellular and fibrous
10/31/27. 8th day of intoxication		0	500	811	2.8	1.1	Present	No change	5	7.85	49	120	4.8	
11/3/27. 11th day after uranium		0	500	400	0.8	0	0	Few granular. Numerous hyaline	10	7.95	45	90	4.4	Following 2nd subcutaneous injection of uranium there developed increase in processes of degeneration of convoluted tubule cells which are normal for this location. Cells show advanced edema and necrosis. Regenerated flattened type of epithelium which has lined many convoluted tubules as process of repair following 1st uranium injection is resistant to 2nd injection of uranium. Such cells show no evidence of degeneration
11/11/27. 19th day after uranium		0	500	593	Present	0	0	No change	55	7.95	30	60	3.8	
11/11/27	17.8 kg.	2	500	Data from the second intoxication by uranium nitrate.										
11/13/27. 2nd day of 2nd intoxication		0	500	641	1.2	Trace	Trace	Increase in granular casts. Numerous hyaline	38	7.95	33	68	4.1	

11/17/27. 6th day of 2nd intoxication		0	500	748	0.8	0	0	No change	44	8.0	33	70	4.0
1/7/28. 1 mo. and 27 days after 2nd intoxication		0	500	463	0.6	0	0	Few granular. Numerous hyaline	63	8.0	32	60	1.7
1/19/28. 2 mos. and 8 days after 2nd uranium		0	500	533	Present	0	0	Occasional finely granular. Numerous hyaline	44	8.0	22.5	44	2.2
1/25/28. 2 mos. and 14 days after 2nd uranium		0	500	786	Present	0	0	No change	33	8.0	28	49	3.0
2/10/28. 2 mos. and 30 days after 2nd uranium		0	500	613	Present	0	0	No change in type of cast. Decrease in number	50	8.0	16	40	2.1

aided by the formation of the low, flattened type of cell which has been previously described. Associated with the specialized type of epithelial repair and in the absence of structural changes of repair in the glomeruli, the animal with the exception of a reduced phenolsulphonephthalein elimination returned to a normal functional state (Fig. 2).

On the 26th day from the commencement of the initial uranium intoxication, the dog was given a second subuctaneous injection of 2 mg. of uranium nitrate per kilo. During this period the animal's weight had undergone a reduction of 1.6 kg. On the 4th day following the second injection of uranium there was an output of 381 cc. of urine which contained 2.3 gm. of albumin and numerous coarsely and finely granular casts. The elimination of phenolsulphonephthalein was reduced to a trace and the reserve alkali was reduced to 7.8. There was a retention of urea of 86 mg. non-protein nitrogen 172 mg. and creatinine 4.8 mg. On the 7th day of the acute nephritis following the second injection of uranium the formation of urine was reduced to 105 cc. which contained 1.1 gm. of albumin. There was no elimination of phenolsulphonephthalein, the reserve alkali of the blood was reduced to 7.7 and there was a retention of urea of 105 mg., non-protein nitrogen 214 mg. and creatinine 6.8 mg. The animal was semiconscious, in air hunger and was killed. The kidneys were swollen and the capsule not adherent. On section the cortex was pale in contrast with the medulla. Histologically the glomeruli varied in their reaction. Many of these units appeared normal except for an intense congestion of the capillaries and a prominence of the endothelium. In others, there was a matting together of the loops, an increase in nuclei, some thickening of the capsule and an early formation of connective tissue. The changes in the proximal convoluted tubule epithelium were characterized by edema, vacuolation and extensive necrosis. There was on evidence of repair. The epithelium of the descending limb of Henle's loop appeared normal (Fig. 3).

In this series of animals subjected to a second injection of uranium nitrate after they had returned to a functional normal which was characterized histologically by a process of epithelial repair with the formation of a type of epithelium closely resembling that of normal convoluted tubule epithelium and in which there was no replacement of these cells by a flattened atypical type, the animals have failed to show any resistance to uranium. On the contrary, judging from the severity of the functional response, as well as from the histological changes in the kidney, they appear to have developed an increased susceptibility to the nephrotoxic agent.

The third and final group of fifteen animals for which the preceding groups served as controls were given a second injection of uranium after they had developed an acute nephritis and had later repaired this

injury with the establishment of a chronic nephritis. When the animals had developed such a state functionally tissue was removed from the left kidney to establish its existence histologically and to serve as a control for those changes developing after the second injection. The animal of Experiment 3, Table IV, shows a response characteristic of this group.

On the 8th day of the acute nephritis there was an output of 811 cc. of urine which contained 2.8 gm. of albumin per liter and granular casts. The elimination of phenolsulphonephthalein was reduced to 5 per cent and the reserve alkali to 7.85. There was a retention of blood urea of 49 mg., non-protein nitrogen 120 mg. and creatinine 4.8 mg. On the 11th day the functional evidence of renal injury had shown an improvement. On the 19th day the animal formed 593 cc. of urine which contained albumin and finely granular and hyaline casts. The elimination of phenolsulphonephthalein had increased to 55 per cent, and the reserve alkali to 7.95. There was evidence of retention with a blood urea of 30 mg., non-protein nitrogen 60 mg. and creatinine 3.8 mg. Such changes in function at this period of recovery from the acute nephritis have been characterized histologically in tissue removed at this time by the commencement of changes of structural repair in the glomeruli and by the regeneration in the injured convoluted tubules of two types of epithelium, either a cell which closely resembles the normal convoluted tubule cell or by a flattened imperfectly differentiated cell layer. On the same day that the above observations were made this animal was given a second subcutaneous injection of 2 mg. of uranium per kilo. On the 2nd day of the secondary acute renal injury the output of urine was 641 cc., and contained 1.2 gm. of albumin and an increase in the number of granular casts. The elimination of phenolsulphonephthalein was only reduced from the animal's pathological normal of 55 per cent to 38 per cent, the reserve alkali of the blood was unaffected and there was but a slight increase in blood retention over that observed at the animal's maximum point of recuperation from the first uranium. On the 6th day of the second intoxication from uranium the amount of albumin in the urine had decreased to 0.8 gm. per liter, the output of phenolsulphonephthalein had increased to 44 per cent, the reserve alkali of the blood was normal and the evidence of retention had diminished. From this stage of the experiment until its termination 3 months after the second uranium injury, there was in general continued evidence of functional improvement. At the final observation there was an output of 613 cc. of urine which contained albumin and finally granular and hyaline casts. The elimination of phenolsulphonephthalein was 50 per cent, the reserve alkali of the blood was normal, there was no retention of blood urea and non-protein nitrogen but a retention of 2.1 mg. of creatinine.

The pathological study showed a definite, chronic, fibrous glomerular injury with the formation of periglomerular and intertubular connective tissue. The epithelial reaction showed evidence of degeneration in the convoluted tubule

TABLE V.

Experiment 7. Duration of Experiment, 4 Months and 12 Days.

Data from the first intoxication by uranium nitrate.

Duration of experiment. Date	Weight. Age	Uranium nitrate	Water intake 24 hrs.	Urine output 24 hrs.	Albumin per liter	Glucose	Acetone	Casts	Phenolsulphone-phthalein	Reserve pH	Urea per 100 cc. blood	Non-protein nitrogen per 100 cc. blood	Creatinine per 100 cc. blood	Pathology of kidney
		mg. per kg.	*cc.*	*cc.*	*gm.*	*per cent*			*per cent*		*mg.*	*mg.*	*mg.*	
Normal observations 11/14/27	18.1 kg. 1–2 yrs.	0	500	483	0	0	0	0	75	8.1	16	33	1.8	Tissue removed from left kidney 1 mo. and 26 days after commencement of nephritis shows glomeruli in various stages of connective tissue overgrowth
11/14/27		2	500											
11/16/27. 2nd day of intoxication		0	500	792	3.0	0.3	Present	Few hyaline. Numerous granular	20	7.9	24	52	2.0	Many capillary loops are obliterated by such changes while others contain blood. Capsules show thickening.
11/21/27. 7th day of intoxication		0	500	811	4.0	0.5	0	No change	5	7.85	97	275	5.4	Formation of periglomerular and intertubular connective tissue. Epithelium of tubules shows cloudy swelling, edema and necrosis of cells in tubules which have not been repaired. In other tubules repair has taken place by formation of type of flattened cells resistant to uranium
11/28/27. 14th day of intoxication		0	500	614	3.2	0.5	0	No change	20	7.9	160	310	7.5	
12/7/27. 24th day of intoxication		0	500	510	1.1	0	0	Decrease in granular and increase in hyaline casts	50	7.95	40	60	3.0	
12/14/27. 1 mo. after intoxication		0	500	723	Present	0	0	No change	45	7.95	42	60	2.8	Changes in kidney which have resulted from 2nd injection of 4 mg. of uranium nitrate per kg. similar to those described for animal of Experiment 3, Table IV Processes of degeneration in epithelium

1/9/28. 1 mo. and 26 days after intoxication	Kidney tissue removed	0	500	583	Present	0	0	No change	60	8.05	31	61	2.8	normal for convoluted tubules intensified. Regenerated epithelium of flattened, non-specialized type unaffected by 2nd injection of increased amount of uranium
1/14/28	17.7 kg.	4	500	Data from the second intoxication by uranium nitrate.										
1/16/28. 2nd day of 2nd intoxication		0	500	748	1	0.6	0	Hyaline casts. Increase in granular casts	51	8.0	22	78	3.0	
1/20/28. 6th day of 2nd intoxication		0	500	914	1	0.5	0	No change	12	8.0	29	72	3.6	
1/26/28. 12th day of 2nd intoxication		0	500	542	Present	0	0	Hyaline casts. Decrease in granular casts	31	8.05	19	47	2.1	
2/26/28. 1 mo. and 12 days after 2nd intoxication		0	500	811	Present	0	0	No change	20	8.1	18	45	2.4	
3/26/28. 2 mos. and 12 days after 2nd intoxication		0	500	510	Present	0	0	Few granular casts. Numerous hyaline casts	53	8.1	17	52	1.8	

epithelium either primarily normal for this location or regenerated after the first uranium injury. The regenerated flattened type of epithelial cell which lined many of the convoluted tubules resulting as a repair process from the initial injury, showed no indications of degeneration. Such cells stained uniformly, and the nuclei were prominent and hyperchromatic. This is the type of cell which is resistant to uranium and which gives to the kidney its so called immunity to a second uranium injury.

The response obtained from the second injection of uranium in this animal is entirely different both functionally and histologically from the preceding animal of Experiment 21, Table III, Figs. 2 and 3. This animal following a less severe acute nephritis returned to a functional normal which was associated histologically by the repair of the acute tubular injury with the formation of an epithelium closely resembling that of normal convoluted tubule epithelium. Following the second use of uranium the animal developed an acute nephritis which was functionally more severe than the initial nephritis. There was extensive necrosis of the regenerated, specialized convoluted tubule cells. The animal was killed after having developed a state of semiconsciousness and air hunger. The specialized type of convoluted tubule epithelial repair in this animal had no resistance to a second administration of uranium. The non-specialized type of convoluted tubule epithelial repair developed by the animal of Experiment 3, Table IV, was resistant to the second injection of uranium.

In order to emphasize the resistance which a certain type of repaired kidney develops to uranium, three animals of this final group were given an acute nephritis from 2 mg. of uranium per kilo and permitted to effect a recovery with the establishment functionally of a chronic nephritis. The character of the anatomical process in the kidney was determined at such a stage of functional improvement by the removal of a piece of renal tissue for study. Following this, the second injection of uranium was given, in which the amount was increased to 4 mg. per kilo. The animal of Experiment 7, Table V, is representative of the findings in these animals.

The height of the dog's reaction from the first injection of uranium was obtained on the 7th day. At this period the animal formed 811 cc. of urine, which contained 4 gm. of albumin per liter, a few hyaline and numerous granular casts. The 2 hour output of phenolsulphonephthalein was reduced to 5 per cent, the reserve

alkali of the blood to 7.85 and there was a retention of blood urea of 97 mg., non-protein nitrogen 275 mg. and creatinine 5.4 mg. Later in the acute nephritis there was an even higher retention of these bodies. On the 24th day of the acute injury there was definite evidence of functional improvement. The urine contained 1.1 gm. of albumin and the number of granular casts had decreased while the hyaline type of cast was definitely increased. The elimination of phenolsulphonephthalein had increased to 50 per cent, the reserve alkali to 7.95, the retention of urea to 40 mg., non-protein nitrogen to 60 mg. and creatinine to 3 mg. 1 month and 26 days following the commencement of the acute injury the animal had reached as indicated by previous studies what appeared to be its maximum degree of functional improvement. On this day a wedge of tissue was removed from the upper pole of the left kidney. The tissue showed chronic glomerular injuries as indicated by the capsular and intracapillary formation of connective tissue with the obliteration of capillary loops and with the formation of adhesions between the loops and the capsule. Other capillary loops were patent and engorged with blood. There was a formation of both periglomerular and intertubular connective tissue. The epithelium of the tubules showed cloudy swelling, edema but rarely necrosis in those tubules in which a repair had not taken place or in the tubules in which repair had developed by the formation of specialized cells resembling normal convoluted tubule cells. In other convoluted tubules a predominant type of repair had taken place by the formation of the flattened type of cell which has been shown to be resistant to uranium (Fig. 4). 5 days after securing this tissue for study, the animal was given 4 mg. of uranium nitrate per kilo, nearly twice the total amount of the primary injection. The dog had decreased 0.3 kg. in body weight. On the 2nd day following the second injection of uranium the animal formed 748 cc. of urine which contained only 1 gm. of albumin. There was an increase in the number of granular casts. The elimination of phenolsulphonephthalein was but slightly decreased from the pathological normal of recuperation of 60 per cent to 51 per cent. The reserve alkali was reduced from 8.05 to 8.0. There was no appreciable increase in the retention of urea, non-protein nitrogen and creatinine. On the 12th day of the secondary acute nephritis the urine contained a heavy trace of albumin, there was a decrease in the number of granular casts, an elimination of phenolsulphonephthalein of 31 per cent and the reserve alkali was 8.05. There was a slight retention of non-protein nitrogen and creatinine. The experiment was terminated 2 months and 12 days following the secondary use of uranium at which time, with but slight variations, the functional state of the dog had remained unchanged for 3 weeks. The 510 cc. of urine obtained on this day contained a heavy trace of albumin, a few granular and numerous hyaline casts. The output of phenolsulphonephthalein was 53 per cent, the reserve alkali was normal. There was no retention of blood urea, a retention of non-protein nitrogen of 52 mg. and no retention of creatinine.

The kidneys at autopsy showed an irregular surface, with a thickened and in

areas adherent capsule. The cortex was irregularly thinned. The glomeruli showed an increase in the degree of fibrosis over that which had been observed following the changes of repair secondary to the first acute nephritis from uranium. The convoluted tubules showed three types of changes. Many of the tubules were lined by the flattened, resistant type of epithelium which has been previously described. In such areas there was no evidence of degeneration from the second injection of uranium which was given in twice the original amount. In other convoluted tubules there was evidence of cell degeneration of the specialized cells normal for the convoluted tubules and repair by the flattened type of cell. Lastly, there were convoluted tubules in which there was no evidence of repair but edema and vacuolation of the epithelium (Figs. 5 and 6.).

These last experiments show conclusively the resistance of the kidney to a secondary nephrotoxic action from uranium to be dependent upon the ability of the kidney to repair the initial epithelial injury by the predominant formation of an atypical epithelium for this part of the tubule and not by the regeneration of a specialized cell closely resembling those which are normally found in the proximal convoluted tubules.

DISCUSSION AND CONCLUSIONS.

The general subject of renal repair has been considered to a greater or less extent in many of the studies concerned with the experimental production of an acute uranium nephritis, as well as those in which the delayed injury was investigated. In 1911 in a study of the regeneration of renal epithelium in the cat kidney (7) two types of processes were described. In the more common type there was an invasion of the necrotic material in the damaged tubules by nuclei which had arisen from not too severely damaged cells in this location which later acquired their cytoplasmic covering. In the more usual type of regeneration a syncytial-like tongue or bud of poorly staining cytoplasm containing hyperchromatic nuclei was described as arising from cells below the area of necrosis, pushing itself into the necrotic material of the tubule, with a differentiation of the cells which arranged themselves on the basement membrane of the tubule. In later studies (8, 9) the regeneration of epithelium in the convoluted tubules was discussed as arising from a similar syncytial ingrowth into these tubules which originated from the cells in the upper portion of the

descending limb of Henle's loop. The origin of such epithelial ingrowths was first observed by Suzuki (10). In a later paper Oliver (11) described the repair to the epithelium as being due to a wandering into the epithelial detritus of cells which became fixed in the area of degeneration and lead to a repair of the tubules. In a very recent study by Hunter (12) of the acquired resistance of the rabbit kidney to uranium, he considers that the epithelial repair originates from convoluted tubule cells which though definitely altered in appearance, remain alive and attached to the basement membrane. From the studies of repair to the tubules which have been made in the preceding paper as well as in the present investigation, in which in addition to the histological studies, functional observations were included, it is clear that more than one type of repair process may be instituted to replace the loss of proximal convoluted tubule cells. If the initial injury from uranium to the epithelium of these tubules is not too severe as is shown functionally in terms of the severity of the response and the rapidity with which the animal establishes a functional normal, the repair is effected by a regeneration of cells from preexisting tubule cells normal for this location (Fig. 7). Such cells have no resistance against a secondary nephrotoxic action from uranium. On the contrary, as has been shown for cells in this location during the gestation period of the dog, when uranium was used to induce an acute nephritis (13), their susceptibility to the poison may be increased. The present studies have confirmed the observation made first in this laboratory and also by Suzuki and Oliver, that a repair to the convoluted tubules may originate as an ingrowth from below of cells as such or in the form of a syncytial mass into the necrotic material in the proximal convoluted tubule (Fig. 8). There is a replacement of convoluted tubule cells in this instance by an entirely different type of cell and following such a replacement the kidney does not return to a functional normal unless at the same time that this second type of repair is developing there is also taking place to a considerable degree a repair by the first process from preexisting convoluted tubule cells. The flattened type of epithelium originating as an ingrowth from below is resistant to uranium, is less specialized in structure and apparently in function.

CONCLUSIONS.

The present study permits the following conclusions.

1. Uranium nitrate in the dog has a high degree of selective affinity as a nephrotoxic for the epithelium of the proximal convoluted tubules. The glomeruli participate in the injury and develop obliterative connective tissue changes as evidence of a preexisting acute injury followed by repair.

2. The repair to the degenerated tubular epithelium is accomplished by two processes. First, by a regeneration of convoluted tubule cells from such cells not too severely injured in this location. This type of regenerated epithelium has no resistance to uranium. Second, the regeneration may occur as an ingrowth of cells or as syncytial buds from cells in the terminal portion of the proximal convoluted tubule or from the upper end of the descending limb of Henle's loop. This type of regenerated epithelium which is entirely different cytologically from normal convoluted tubule epithelium is resistant to a second injury from uranium even when the amount of this nephrotoxic agent has been increased to double the amount of the initial injection.

3. The kidney does not develop a local tissue immunity or resistance to uranium in the sense that cells of the same type once injured by it acquire as a result of the injury a resistance. The resistance and apparent but not real immunity is due to another type of cell with resistance having been substituted for a cell with but little resistance. This fact may be looked upon as constituting part of a defense mechanism in the kidney and may in part explain the long duration of certain types of chronic nephritic processes.

4. The functional studies which have been made during the initial injury from uranium to the tubules and during the secondary injury in animals which have either shown a resistance or a lack of resistance, emphasize the importance of the tubular epithelium as a part of a secretory mechanism in urine formation. During periods when the proximal convoluted tubule epithelium is in a state of acute degeneration there is a disturbance in the acid-base equilibrium of the blood, a reduction in the elimination of phenolsulphonephthalein and a retention of urea nitrogen, non-protein nitrogen and creatinine. When this epithelium is regenerated by the formation of a tubular epithelium

normal in character for this location of the tubule, regardless of structural changes in the glomeruli, the above evidence of renal dysfunction returns to the normal. If at such a period this type of regenerated epithelium be injured by a secondary injection of uranium a state of acute renal dysfunction is induced in an intensified form. In those animals in which the repair to the tubules was accomplished by the formation of an atypical type of epithelium in the convoluted tubules as well as by the formation of cells normal in histological appearance for this part of the tubule there was an improvement in the degree of depletion of the reserve alkali of the blood, in the elimination of phenolsulphonephthalein and in the retention of urea nitrogen, non-protein nitrogen and creatinine. Certain of these values did not reach the normal. In such a state of renal repair when a second injection of uranium was given the kidney was found to have developed a marked resistance to it. There was but slight evidence of a depression in renal function. Associated with this acquired functional resistance there was no evidence of injury to the atypical, flattened regenerated epithelium of the proximal convoluted tubules.

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EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the control animal of Experiment 11, Table II. The second injection of uranium was given during the initial acute nephritis before there was any evidence of an improvement in renal function. A secondary injection of uranium at such a period increases the severity of the acute pathology and intensifies the evidence of functional impairment. The kidney shows no resistance to uranium. At *a*, is shown a glomerulus with the capillaries distended with blood. The endothelial cells are prominent with deeply staining nuclei. There is no subcapsular exudate. At *b*, are shown convoluted tubules in various stages of edema and necrosis. At *c*, is shown the descending limb of Henle's loop and at *d*, the ascending limb with a normal epithelial lining.

FIG. 2. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the control animal of Experiment 21, Table III. The tissue was removed from the kidney after the animal had made a return to a normal functional state following the initial acute nephritis from uranium. The figure shows at *a*, a large glomerulus with capillaries engorged with blood and endothelial cells with hyperchromatic and hyperplastic nuclei. The capsule is not thickened and there is no periglomerular formation of connective tissue. Many of the convoluted tubules at *b*, show a regeneration of epithelium normal in type for this location of the tubule. In such tubules at *c*, are shown mitotic figures. In other convoluted tubules at *d*, the epithelium shows evidence of degeneration and imperfect repair.

PLATE 35.

FIG. 3. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the same animal, control Experiment 21, Table III, after the animal had received a second injection of uranium. The figure shows at *a*, a glomerulus with an increase in connective tissue nuclei and an early thickening of the capsule. At *b*, there has occurred an early formation of intertubular connective tissue. At *c*, is shown the convoluted tubule epithelium in an advanced stage of edema, vacuolation and necrosis. The cells of the loops of Henle at *d*, appear normal. The experiment shows that a regenerated type of convoluted tubule epithelium normal for this part of the tubule has no resistance to a secondary injection of uranium. Such cells rapidly become necrotic and renal function is severely depressed.

FIG. 4. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 7, Table V. The tissue was removed from the kidney after the animal had reached its maximum degree of functional improvment from the first injection of uranium with the development of a chronic nephritis. The figure shows at *a*, convoluted tubules in which epithelial repair has taken place by the regeneration of cells normal for this location. Evidence of degenera-

tion is found in some of these areas. At *b*, is shown the dominant type of epithelial repair by the formation of an epithelial lining for the convoluted tubules by a flattened type of cell atypical for this location. At *c*, is shown a marked formation of intertubular connective tissue.

PLATE 36.

FIG. 5. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is also from the animal of Experiment 7, Table V, after the second injection of twice the amount of uranium used at the initial injection. The figure shows at *a*, regenerated convoluted tubule epithelium normal for this location in the process of an acute degeneration. Other cells show evidence of repair. This type of epithelium is not resistant to a second injection of uranium. At *b*, is shown the flattened, regenerated type of epithelium which is atypical for this location in the tubule. Such cells show no evidence of injury from the second injection of uranium. There was but slight evidence of any impairment of renal functions from the second injection. At *c*, is shown a diffuse formation of intertubular connective tissue.

FIG. 6. Camera lucida drawing, Leitz, oc. 2, obj. 6. The figure is also from the animal of Experiment 7, Table V. It shows at *a*, a glomerulus in an advanced stage of connective tissue formation and at *b*, and *c*, areas of periglomerular and intertubular connective tissue formation. At *d*, are shown convoluted tubules lined by the flattened type of atypical epithelium which has developed no evidence of injury from the second injection of uranium. This type of epithelial repair is responsible for the resistance which the kidney develops to a second injection of uranium. At *e*, are two small arteries showing a thickening of the middle coats.

PLATE 37.

FIG. 7. Camera lucida drawing, Leitz, oc. 2, obj. 1/12. The figure is from two areas in the kidney of the animal of Experiment VII, Table V. At *a*, is shown a convoluted tubule in which epithelium normal in character for this portion of the tubule is in the process of regeneration. Mitotic figures are present. At *b*, these tubules are being relined by the flattened resistant type of atypical epithelium. The tubules contain casts. At *c*, is shown part of a glomerulus with fibrous tissue formation, capsular thickening and adhesions between the capillaries and the capsule. At *d*, is shown one of the smaller renal arteries with a thickened middle coat while *e*, shows the diffuse formation of intertubular connective tissue.

FIG. 8. Camera lucida drawing, Leitz, oc. 2, obj. 1/12. The figure is from the kidney of the animal of Experiment 7, Table V. It shows at *a*, a proximal convoluted tubule with epithelium edematous, vacuolated and necrotic. At *b*, are shown convoluted tubules in which the necrotic material has been removed and which have been relined by the flattened, atypical type of cell. At *c*, are shown cells of this type in stages of mitotic division.

FIG. 1.

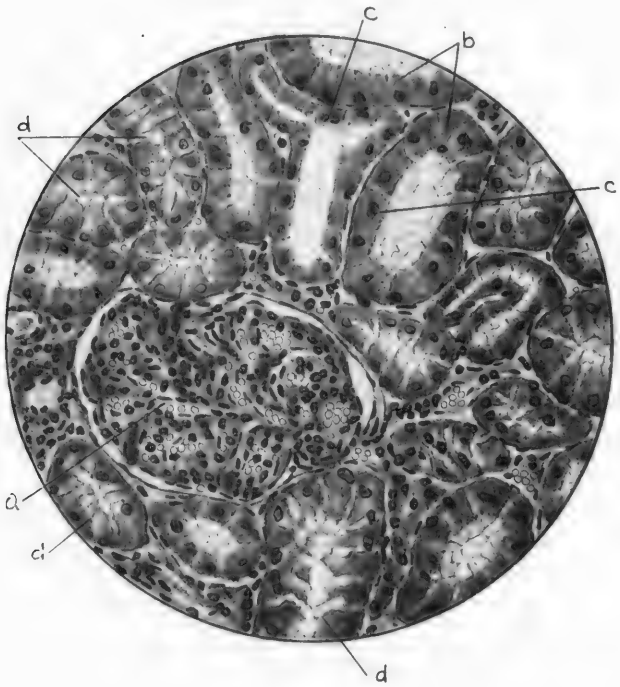

FIG. 2.

(MacNider: Kidney response to uranium nitrate.)

FIG. 3.

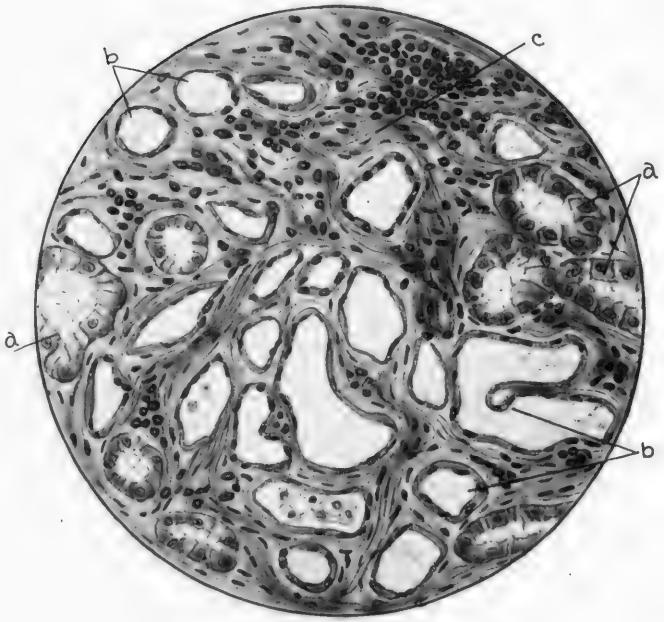

FIG. 4.

(MacNider: Kidney response to uranium nitrate.)

FIG. 5.

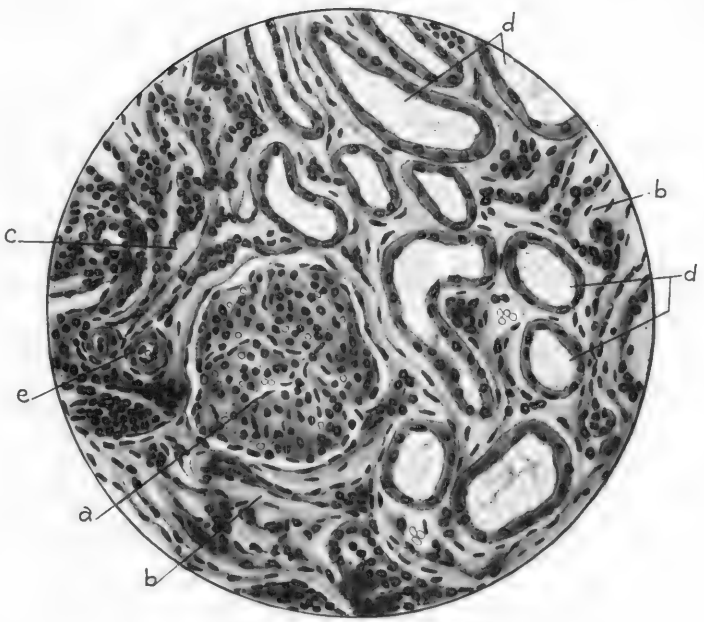

FIG. 6.

(MacNider: Kidney response to uranium nitrate.)

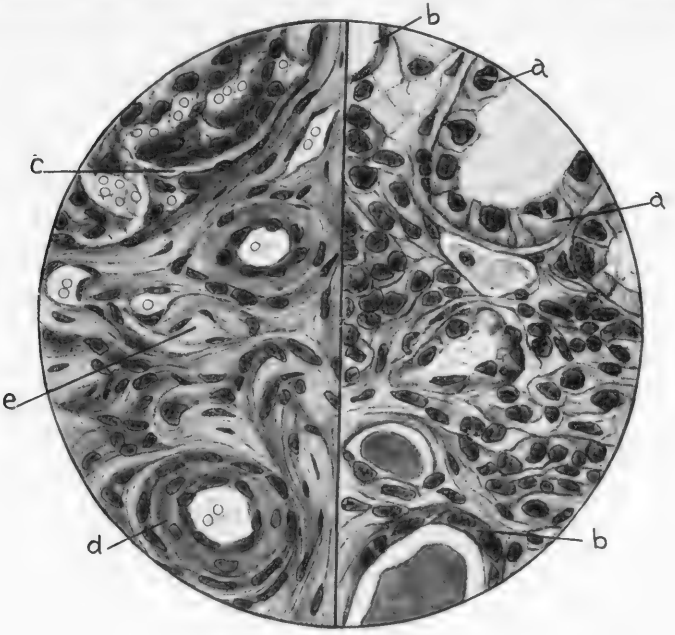

FIG. 7.

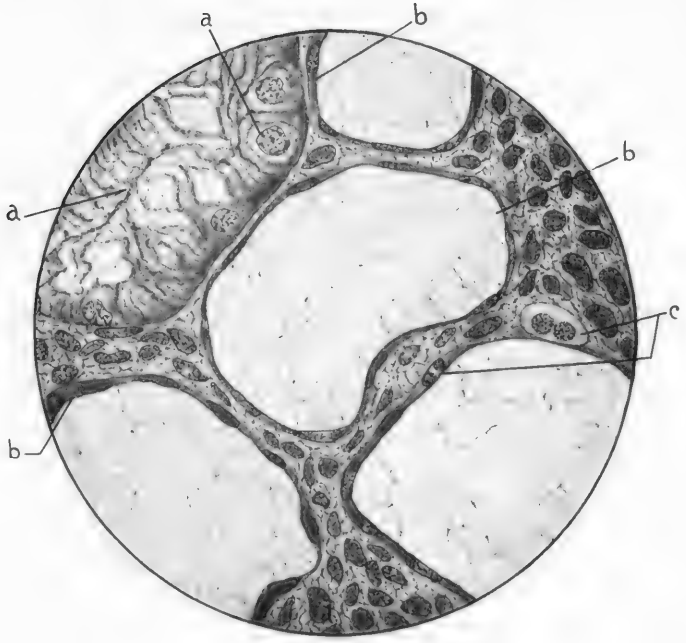

FIG. 8.

(MacNider: Kidney response to uranium nitrate.)

UNIV. OF MICH.

OUTLYING ACIDOSIS DUE TO FUNCTIONAL ISCHEMIA.

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In a note from this laboratory published some little time ago (1) the occurrence was reported of states in which a frank acidosis of the tissues develops without any change in the reaction of the blood. The purpose of the present paper is to describe more fully this outlying acidosis and to consider its causes.

Most of the blood acidoses encountered by the clinician arise through tissue activities, needless to say—those referable to acid materials taken by mouth being highly exceptional. Inevitably the aim of students of acidosis must be to determine the where and the how of its production by the organs. Despite the perfecting of methods for the study of blood states and of the excreta we are still, in respect to this problem, much like explorers unable to penetrate into a continent, and gaining information of the life there only by the observation of what is borne on and out by its great rivers.

Local Acidosis Due to Mechanical Interference with the Circulation.

The fact that under circumstances of deficient oxidation tissues form acid substances has long been commonplace. One would expect that where the circulation is seriously impaired both these and the acid products of ordinary cell activities will tend to be retained. Experiments upon animals vitally stained with indicators have demonstrated that the accumulation under such circumstances is frequently sufficient to affect the tissue reaction perceptibly. Vigorous skin grafts are relatively acid during the days before they become vascularized (2). In a normal rat vitally stained with phenol red interruption of the circulation to a leg by means of a tourniquet leads within a few minutes to a change in the color of the limb from red to orange-yellow. These are simple instances of acidosis in the superficial

tissues. We have carried out ligation experiments to determine whether evidence can be had elsewhere of a similar state of affairs.

Experiment I. The Local Manifestations of Acidosis on Direct Interference with the Circulation.—White rats were employed which had been shaved where necessary for the observations and stained with phenol red or brom cresol purple. The methods of staining by intraperitoneal injection, and of inspection have been fully described in previous papers (3–5). The shaving had been skilfully done some days beforehand. In order to minimize the loss of carbon dioxide from organs exposed during the course of the work the animals were submerged in paraffin oil kept at body temperature (5). Sections of the parenchymal tissues were made under oil with the Valentine knife, normal portions of the same organ, or of its fellow in the case of paired organs, serving as control. They were examined immediately, between mica slides.

In brief it was found that when the circulation to an extremity had been interrupted the reaction of the subcutaneous tissue undergoes a manifest alteration toward acidity within a few minutes, the alteration being sufficient to change the color, not merely of phenol red, but of brom cresol purple (from purply blue to blue-green). A similar change of brom cresol purple occurs even more rapidly in the skeletal muscles. In them, though, the alteration is traceable to that part of the phthalein which is held in the tissue fluids, a fact readily to be demonstrated in the long muscles of the leg. The belly of one of these muscles in which the circulation has been stopped for some minutes appears greenish purple when viewed *in situ* with a piece of white card slipped under it as background, whereas the control muscle in the unaffected leg is clear purple. But if the two are gently compressed between glass slides to drive fluid out of them they take on almost precisely the same purply green hue. This might have been predicted; for the muscle fibers themselves stain a light greenish yellow with brom cresol purple even under ordinary circumstances (4), a hue so far toward the acid end of the indicator range that any change toward acidity which might occur in such fibers would be unaccompanied by any pronounced change in the general color,—this last being, of course, composite in make-up.

The color change occurring in portions of the spleen with ligated vessels is likewise confined almost wholly to the phthalein dissolved in the tissue fluids. The organ stains but poorly with the indicator.

The tissues of liver and kidney do not become evidently more acid to brom cresol purple until 15 or 20 minutes after the circulation has been stopped; but the reaction of their parenchyma is then definitely affected as shown both by observation under the microscope and by the pressure test. The small intestine becomes acid somewhat more rapidly. On the other hand, the reaction of the tendons, and of fascia at a distance from muscle, as for example in the lower leg or tail, changes not at all during a half-hour or more of circulatory obstruction.

These observations, while fragmentary, suffice to bring out significant differences in the degree of color change, as indicative of acidosis, in various organs deprived of their circulation. The fact that no alteration occurs for a long time in tendon and fascia is in keeping with what is known of the metabolic rate of these tissues. On the basis of relative rates a much more prompt alteration had been expected in the kidney and liver than in the subcutaneous tissue. But as a matter of fact a change was regularly evident within this tissue after only about a third of the time required for the development of one in the parenchymal organs mentioned. The fact may be recalled in this connection that the injection of acid or alkali into the circulation fails to alter the reaction of the liver, even when the procedure is pushed to the limit of tolerance and the reaction of the blood and matrix tissues (tendon, connective tissue, cartilage) has been pronouncedly altered (5). Very probably the hepatic cells, and quite possibly cells in general, maintain their reaction so long as they are uninjured, the acid products of their metabolism being cast out into the surrounding fluid or segregated within the protoplasm.

Because of the difficulties of interpretation brought out by the foregoing experiment, as well as for obvious reasons of expediency, it seemed best to begin the study of outlying acidosis with observations on the superficial connective tissue.

Local Acidosis Due to Epinephrin Ischemia.

A type example of local acidosis from functional ischemia, that which follows upon the subcutaneous injection of epinephrin, has been investigated at length.

Experiment II. The Local Changes in Reaction Caused by Epinephrin.—As in the previous experiment, white rats were used, vitally stained by the injection of phenol red or brom cresol purple into the peritoneal cavity. Sometimes the animals were anesthetized by the injection of a urethane solution into the tissues of the back of the neck (5). In such case they were laid upon an electrically warmed pad and the shaved body surface was swabbed with paraffin oil, a procedure which brings out brilliantly the hue of the stained tissues. Several specimens of 1:1000 adrenalin chloride (Parke, Davis and Company) were employed. These proved acid to phenol red, and were brought to pH 7.4 just prior to use by titration with N/10 NaOH in the presence of a trace of the indicator. Control tests on stained animals demonstrated that the amount of chloretone present in the epinephrin preparations had no effect to alter the local tissue reaction.

A fraction of a drop of the epinephrin solution introduced through a fine needle into the subcutaneous abdominal tissue of unstained rats, well to one side of the midline, caused blanching along the needle track in 7 to 10 seconds, and this blanching spread until, within the course of a few minutes, there existed a pallid patch 2 or more cm. broad. In animals stained with phenol red on the other hand, no swift blanching or other color change occurred along the needle track,—fresh evidence in addition to that already provided (3) that the staining is essentially extravascular. After about 2 minutes, however, an alteration from red toward yellow could be noted at the site of injection; and within 5 minutes there was present a well demarcated orange patch of the same dimensions as the blanched patch to be seen at this time in unstained animals. During the next half-hour this patch persisted and often spread somewhat, the color becoming a marked orange-yellow. Meantime the staining of the animal as a whole frequently became less intense, owing to elimination of the dye; but this process failed to affect the color of the patch which in consequence soon came to stand out boldly on a pink background.

The first sign of a wearing off of the effect of the adrenalin was a reversion from orange to red at the margin of the patch. Occasionally a rosy spot developed at its center as well. When the newly reddened tissue was compressed between slides its aspect did not alter immediately,—again showing the extravascular situation of the dye,—but after a few minutes of such compression it turned yellow, becoming red once more when released. These changes were very vivid when they occurred, as not infrequently, in rats that had decolorized everywhere else. Under these circumstances the marginal tissue after turning red soon decolorized too, and the patch dwindled in size by a peripheral reversion from yellow to red, followed by removal of the dye. The time from epinephrin injection to complete disappearance of the patch was usually almost 2 hours.

That ischemia was the cause of the local acidosis responsible for the color change became clearly evident when the epinephrin injection preceded that of the phenol red by 5 minutes or more. Then one saw, as the animal became vitally stained, that none of the dye entered the areas blanched by the epinephrin, the result being that they stood out as pallid blotches against surroundings that had rapidly colored an intense red with the highly diffusible phthalein. For as long as a half-hour this phenomenon persisted, and then, as the adrenalin effect wore off, color crept in gradually at the edges and sometimes at the center of the pale blotches; and where this took place the tissue was at first stained orange-yellow, turning orange later and then red.

Brom thymol blue could not be utilized in the attempt to gauge the acidity of the ischemic patches because it is not only toxic but gives false indications *in vivo* (4, 6); and no other indicator with its range has been found satisfactory. Evidence corroboratory to that with phenol red could, however, be obtained by vital staining with brom cresol purple. The subcutaneous tissue of the rat has a reaction which causes it to take on a hue about midway in the rather wide and but slightly various purple portion of the range of the indicator (5). For this reason

considerable alterations in pH are required to bring about a manifest color change *in vivo*. Yet such a change was sometimes apparent in subcutaneous regions rendered ischemic by adrenalin,—one from frank purple to greenish purple. A similar but more pronounced change was visible in the ear of the stained rat, injected with adrenalin and viewed by transmitted light. In the subcutaneous tissue of the abdomen N/100 HCl gave rise to a color change about as great as that due to epinephrin; but N/10 HCl turned the purple to yellow. In animals stained with phenol red the epinephrin patches were never so yellow as those developing elsewhere in the same animals after subcutaneous injection of N/10 HCl. The acidity in the ischemic patches was, in other words, never so great as to lie entirely beyond the range of phenol red, a finding confirmed by the observations with brom cresol purple.

Some specimens of epinephrin caused a greater color change in animals stained with phenol red than did others, whence one may perhaps infer that they had a more intense vasoconstrictor action.

The fact is plain from these observations that temporary ischemia due to a local contraction of the subcutaneous vessels leads within a few minutes to acidosis in the region affected. Much of this can be referred to the accumulation of carbon dioxide,—a factor dealt with in previous papers from this laboratory (2, 3). When the skin is everted, ventilating the patches, their color quickly changes to red and then to purply rose. Carbon dioxide passes through the tissues with such ease (7), and in the rat the tissue layer upon which the epinephrine acts is so shallow,—even when, as sometimes happens, the underlying muscle partakes of the ischemia,—that a not inconsiderable escape of the gas from the patches into their surroundings must be supposed to occur even when the circulation is at a standstill in them. This would tend to mitigate the acidity.

It is possible to gauge with considerable accuracy the apparent pH of superficial tissues stained with phenol red and to measure the extent of the changes taking place in the reaction (8). But that was not attempted in these experiments.

Paradoxical Acidosis.

The foregoing observations justify the belief that in clinical conditions which involve a functional impairment of the circulation to special regions, as *e.g.* in Raynaud's disease, a local acidosis may develop so considerable as to alter the tissue reaction. The occurrence of widely distributed changes in the tissue reaction independent

of blood changes was brought to our attention by a singular and convincing instance. A shaved white rat stained with phenol red had been given several cc. of a strong solution of sodium carbonate by stomach tube. The blood reaction became more alkaline soon after, and alkaline urine was voided; yet the shaved body instead of participating in the alkalinity, as shown by a change in hue of the stained tissue from red to purply rose, turned orange-yellow, became relatively acid that is to say. The phenomenon can be elicited at will.

Experiment III. Outlying Acidosis Caused by Sodium Carbonate.—Shaved rats given no food for 24 hours but allowed water until the time of the experiment were given an intraperitoneal injection of isotonic 4 per cent phenol red at pH 7.4; and, when they were well stained, warmed N/1 sodium carbonate (Merck) was administered by stomach tube in amounts of from 3½ to 10 cc., depending upon the weight of the animal and how extreme were the instances desired. Amounts greater than 5 cc. were given in divided doses since otherwise regurgitation often ensued. The pH of the blood was followed by the aspiration of samples from the heart into short segments of ordinary 1 cc. pipettes graduated in hundredths, which had been ground to fit short aspirating needles of stainless steel. 0.2 cc. of blood was taken at a time, a paraffin seal preventing the entrance of air at the junction of glass and needle during the aspiration. The pH was determined by a modification of Hawkins' method (9), whereby 0.1 cc. of blood was introduced into 2 cc. of salt solution previously tinted with phenol red and brought to pH 7.4 under oil. Mixing was accomplished with a thin glass stirring rod, the oil quickly replaced with paraffin that solidified on cooling, the tube centrifuged, and the color read at 38°C. in an ordinary comparator block. Many tests in duplicate showed that the findings with a single one could be relied upon, as also that the pH of the blood-salt mixture did not change during a period much longer than that which elapsed prior to the readings. The remainder of the blood specimen was utilized for determination of the cell-plasma relationship by Van Allen's method (10) which requires only a fraction of a drop. Here too control tests demonstrated the validity of our technic. It seemed better to repeat the experiment often than to take each time the amount of blood that would have been required for duplicate tests.

The first blood specimen was withdrawn after the vital staining was well established, and thereafter the animal was watched for signs of heart injury during some minutes before the carbonate was given. Usually observations were carried out on several animals at a time, kept on absorbent cotton in separate jars. The blood pH of controls merely stained with phenol red did not vary, nor did the surface color alter.

Unanesthetized rats of about 150 gm., receiving 5 to 7½ cc. of N/1 sodium carbonate, appear uncomfortable within 15 minutes, the hair roughening; and soon they begin to jump intermittently and aimlessly. After 20 minutes to ½ hour in

typical instances the color of the shaved and oiled belly is noted to be changing from red toward yellow. At this time the animal is still on its feet and appears strong. It is now often voiding urine that is alkaline, as shown by the hue of the phenol red it contains, whereas that passed prior to the carbonate was acid by the same criterion. Within an hour the animal is cold and feeble, sitting quiet, or in a state of semicollapse; and its color is an outspoken orange-yellow. Often it dies a little later, with the yellow hue even more pronounced. If it is to survive, an active purgation of semifluid, rose-purple material takes place, together with an alkaline diuresis, and decolorization is complete in the course of 3 or 4 hours. Not infrequently, when only 5 cc. of carbonate is given, no signs of acidosis develop. In our animals that succumbed after administration of the salt the heart was examined for injury from the aspirating needle, and instances in which this might have caused death were ruled from consideration.

The second blood specimen was ordinarily taken when the surface of the body appeared notably orange. There was either no change in pH or else a rise (from pH 7.4–5 to pH 7.6 or even to pH 7.7 in some cases).

To study carefully the progress of the acidosis numerous rats were urethanized, and after the carbonate had been given they were placed in the oil bath (5). In this way the influence upon the phenomenon of chilling of the shaved skin was ruled out. Urethane of itself has no effect upon the pH of rat blood within periods so short as those of our experiments (11). In rats under its influence the blood specimens were taken directly from the common carotid artery. The vessel was dissected free, seized with a mosquito forceps, and the wound cavity filled with oil. The forceps was given a quarter turn around its axis and away from the heart so that the distended vessel lay in a curve over its blades and the needle was inserted at the curve and toward the heart. As the needle was withdrawn, after 0.2 cc. of blood had been obtained, the flow was checked either by turning the clamp a little further, or preferably by traction upon a thread placed loosely about the vessel. A fine ligature was then put on at leisure. It was found that no blood need be lost by the method and that the same vessel can be aspirated several times. The needle employed should have a short bevel, and should be inserted with the bevel side up.

The findings in urethanized rats confirmed those in the unanesthetized; and much less carbonate sufficed to bring about the acidosis.

Relation of Anhydremia to the Acidosis.

The development of tissue acidosis in the rats given sodium carbonate was regularly accompanied by a change in the cell-plasma relationship of the blood, the proportion of cells increasing markedly, sometimes enormously, at the expense of the plasma (from 43.5 to 64.5 per cent; from 44 to 67 per cent,—to cite but two instances out of a large number). The degree of the acidosis was paralleled by

that of the blood change. That this change was the expression of an anhydremia cannot be doubted; for not only was the N/1 sodium carbonate solution strongly hypertonic, but the amount of fluid accumulating out of the body into the bowel was such as to distend it markedly. In certain animals which had taken water shortly before carbonate the ordinary dose of this latter caused no change whatever in the surface hue and there was either no change in the blood plasma relationship or practically none.

There exists a considerable literature describing blood acidosis as a complication of clinical anhydremia (12). In order to find whether anhydremia was the cause of the paradoxical tissue acidosis in our rats receiving carbonate, recourse was had to experiments in which the condition was produced by other means. Active purgation with N/1 magnesium sulfate soon proved effective, as disclosed by hematocrit tests, and there developed with it an acidosis of the superficial tissues. But a gradual respiratory failure took place at the same time and might have been largely responsible for the acidotic change. No such complication was observed with N/1 sodium sulfate. A rat of 138 gm. given 20 cc. of it in the course of 2½ hours, and purging actively as result, stained orange when injected with phenol red an hour later whereas it had stained red at a previous injection. The animal was now semiprostrate with exaggerated respirations. The hematocrit showed that 56 per cent of the blood volume consisted of cells, the normal proportion being between 40 and 45 per cent. The experiment was not repeated since a simpler method to produce anhydremia,—and outlying acidosis,—was found in the administration of hypertonic sugar solutions.

Experiment IV. Anhydremia,—and Outlying Acidosis,—Following the Injection of Hypertonic Dextrose Solution.—The general procedures were identical with those of Experiment III except that 5 to 10 cc. of a 50 in 100 solution in water of Merck's highest purity anhydrous dextrose was administered instead of carbonate; and the symptoms manifested by the rats were practically the same. The change from red toward yellow was swifter, though, and more considerable. The animals frequently died within an hour. Often before collapse occurred the rat was a brilliant orange-yellow, of such a hue indeed as was not attained by stained controls until a half-hour or more after they had been killed (by a blow on the head). Of those which survived some failed to alter in hue or became only slightly or transiently yellower. They purged a thin fluid stained yellow with phthalein.

The same changes in the hematocrit findings occurred as in the carbonate animals, and in the same general parallelism with the degree of tissue acidosis. But whereas in the carbonate instances the reaction of the blood either failed to alter or became more alkaline, in the sugar ones, though occasionally constant, it tended ordinarily to fall somewhat (in the most extreme instance from approximately pH 7.4 to pH 7.1). In rats that survived and manifested slight tissue acidosis or none, the pH of the blood did not change and the cell-plasma relationship was little affected.

In these instances of anhydremia caused by the ingestion of sugar the blood pH ordinarily fell. The change taking place in the superficial tissues might be deemed merely an expression of the blood alteration were it not far more considerable,—from about pH 7.4 to pH 6.4, to judge from the colors, as one is justified in doing (13). In papers already published we have shown that in normal animals changes produced in the reaction of the blood by the direct introduction of alkali or of acids, including H_2CO_3, are closely attended by changes of the same degree in the reaction of the subcutaneous tissue (8, 14). But the alteration occurring in this tissue when sugar has been given by mouth is out of all proportion to that in the blood, and hence it falls in the category of outlying acidosis.

Averting Anhydremia Averts the Acidosis.

In the course of the experiments with carbonate and sugar there had been numerous indications that when the dose of hypertonic material is insufficient to produce anhydremia, or when this last is averted through purgation and diuresis, outlying acidosis fails to occur. In amplifications of such findings some direct attempts were made to prevent the development of anhydremia. The results demonstrated that in proportion as they were effectual so too was acidosis lessened or prevented.

Experiment V. Prevention of Outlying Acidosis by Water Injections.—Ordinarily, for each test, four or six rats of identical weight were employed which had been urethanized and stained with phenol red. The same dose of N/1 sodium carbonate was given to all, and they were paired for intensity of color,—whenever any differences existed,—and laid on the back, side by side, upon the warm pad. A few minutes later the slow injection was begun of warm distilled water into the peritoneal cavity of one animal of each pair, through a hypodermic needle connected with a burette. The blood was followed, by the methods already described, with specimens taken from the carotid.

Clinical experience has shown that it is often difficult to check the course of anhydremia; and our efforts with animals given carbonate were only successful with critical doses of this latter. When more was given the desiccation and acidosis, which developed rapidly, were uninfluenced by water; when less, none of the animals became sufficiently anhydremic and acidotic for the purposes of the work. It would have been necessary under test-tube conditions to have added nearly 4 cc. of water to each cc. of N/1 carbonate in order to render the solution isotonic with the blood. To introduce the needed total into rats was manifestly impossible. But when the carbonate dose had been rightly chosen it was found that the gradual introduction of 10 to 18 cc. of water saved the animals from anhydremia, with attendant tissue acidosis, a condition to which controls succumbed. The fluid was rapidly removed from the peritoneal cavity of the injected rats, there was slight change at the most in the cell-plasma relationship, the blood became more alkaline than is normal, and alkaline purgation and diuresis took place, with rapid decolorization and recovery.

Similar but less extensive experiments in which dextrose was used instead of carbonate gave results that were similar except that the blood of the animals receiving the water did not become more alkaline than normal.

Better results in the prevention of anhydremia could have been obtained, doubtless, by the utilization of salt solution (12); but with the salt there would have been introduced a fresh difficulty in the interpretation of the experiment.

Outlying Acidosis after Hemorrhage.

Anhydremic animals remain stained with phenol red long after the controls have decolorized. This indicates that there is a considerable interference with fluid interchange, as does the fact that the blood fails to become more alkaline when the dose of hypertonic sodium carbonate solution is very large. In the fulminant anhydremia, accompanied by tissue acidosis, resulting from such a dose the passage of fluid is all one way, so to speak,—into the gut. According to the literature the acidosis of the blood encountered in clinical anhydremia can be referred to metabolic disturbances arising from a diminished volume flow through the organs; and this in turn is due to a vasoconstriction compensatory for the lessened blood bulk. If the tissue acidosis is referable to diminished volume flow then it should develop when the blood bulk is diminished in other ways, as for example by hemorrhage. Such is the case.

Experiment VI. Outlying Acidosis as the Result of Hemorrhage.—The methods of staining, examination, etc., were identical with those in the preceding experiments. Rats anesthetized with urethane were bled from the carotid by the method employed to obtain blood samples.

It was soon found that large depletions were required to induce tissue acidosis. For instance in one rat out of a series weighing 136 gm. that were urethanized, fasted 18 hours, and bled repeatedly from the carotid at short intervals, the removal of 0.8 cc. of blood caused only a dubious change in the surface color. In a second animal bled 1.0 cc., the red hue altered definitely toward yellow; while two others losing respectively 1.4 and 1.6 cc. became notably orange, though the blood pH did not alter from its initial value. In the instances last mentioned the hematocrit findings at the time when tissue acidosis was well developed showed that the blood bulk had been but partially made up, the cell percentages having dropped only from 51 per cent to 49 per cent and from 57 per cent to 50 per cent respectively. The animal that lost most blood retained the orange-yellow hue and died some hours later.

Numerous other examples of the sort could be described. Decolorization failed to occur in the bled animals within the usual time, and in extreme instances of depletion involving tissue acidosis the blood pH often fell slightly. If the rats had taken water shortly before they were bled the loss in blood volume was made up more or less completely, and in proportion as this happened acidosis was transitory or failed to occur.

Urethanized animals stand bleeding but poorly and when acid is gradually introduced into the blood stream there is little or no compensatory blowing off of carbon dioxide (8). For these reasons the experiments were repeated in the absence of the anesthetic. Blood was removed by aspiration from the heart. The findings were essentially the same as after urethane.

Here again was a frank tissue acidosis unaccompanied by change in the blood reaction. In those extreme cases in which the blood pH fell, the fall in pH occurring in the tissues was several times more considerable, as disclosed by their color.

Characteristics of the Outlying Acidosis.

The surface changes in hue of the phthalein-stained animal after hemorrhage, or during the development of carbonate anhydremia are of like character. When taking place gradually they exhibit a significant sequence. One notes that the body is becoming irregularly blotched, or mottled, as the result of local alterations from red toward yellow in the hue of the stained tissue. The blotches are ill-defined to begin with, asymmetrical, irregularly oval or rounded, and they vary

less in size than in color with the degree of the depletion. Those seen on the chest, abdomen, and thighs of shaved rats are from ½ to 1 cm. in greatest diameter, scattered upon a red background. They are usually discernible in smartly bled animals some 5 to 10 minutes after the depletion. When the blood bulk is poorly made up they grow progressively larger and more yellow. Meantime the regions between them become less red; and after a greater or less time, by enlargement and coalescence of the patches and by the just mentioned change in color of the interspaces, the orange-yellow hue becomes general. But before this happens the animal is often pronouncedly mottled in orange-yellow against red, a mottling which extends to the tissues still protected by hair as can be shown by stripping the pelt. Where the hair has been shaved off the patches are beautifully seen by transmitted light. Chilling of the skin is not the cause of them. For they occur in animals immersed in warm oil, and fail to develop in stained and urethanized controls placed in the ice box at 0.5°C. and succumbing in the course of an hour or more to the exposure.

If the depletion by hemorrhage has been extreme, or a fulminant anhydremia has been induced, a general acidosis of the surface tissue develops, with scant signs of patching. The time needed for the color changes implicit in the patching is enough in such instances for a diffuse alteration. Patching has never been encountered in rats rendered anhydremic with sugar; and the generalized change in hue occurring in such animals before death is, as already stated, far greater than under ordinary circumstances of diminished blood bulk, the color attained being nearer to yellow. There are, of course, special metabolic conditions to account for this. On the other hand a state of depletion can be induced with carbonate or by bleeding which leads to the development of mottling only, and this may persist for a long time without essential change in the situation or shape of the blotches. In such cases gentle massage will often suffice to bring back the red color to individual patches and obliterate them; but later they reappear. When they are merely a transient stage in the rapid progression to a generalized acidosis massage is without effect.

In animals that are recovering the patches grow smaller by a peripheral encroachment of red, and there goes on within them at the same time a reversion to this hue.

According to Kendall (15, 16) the adsorption *in vitro* of phenol red upon tissues is greatest when they are relatively acid; and we have noted this to be true of some of the other phthaleins as well (13). The factor seems to be without influence on the character of the phenomena witnessed in the living animal and just described. There is, furthermore, no evidence of a special destruction of the dye in regions that are poorly oxygenated. When the pelt is stripped back in depleted animals showing a patchy acidosis the rose-red to which the phthalein attains is as intense where the patches were as elsewhere. Incidentally it should be said that the change to alkalinity thus attested takes place also when the acidosis is generalized throughout the superficial tissue after bleeding or sodium carbonate; but that when sugar has been used as the depleting agent the orange hue is often slow to alter, the color not progressing beyond orange-red.

An Outlying Acidosis Due to Intercurrent Pressure Differences.

In extension of the observations a series of rabbits were depleted by hemorrhage. Elsewhere we have reported on the local acidosis developing in such animals when deeply under the influence of ether or urethane. No such acidosis is met in intact animals properly anesthetized with these agents, but the characteristic patchy mottling can readily be induced in them by bleeding. It was deemed best to avoid the influence of general anesthesia. For this reason a carotid was cannulated under ether some hours or a day beforehand and when the animal was fully recovered it was bled repeatedly, with the aid of novocaine locally. Under such circumstances, as the shaved, stained, and depleted rabbits sat quiet in the ordinary hunched position, a type of acidotic patching was seen to develop wholly distinct from that thus far considered, and preceding it slightly in point of time. This patching was localized to the surface regions overlying the bony prominences (knees, backbone), regions which had colored quite as rapidly as had those elsewhere immediately after the injection of the phenol red, and indeed sometimes in our experience slightly more rapidly. The change could readily be demonstrated by pulling to one side the loose skin from over the spine, for then there was brought to view a broad orange band which had lain over it. Rats are not so well suited to a demonstration of the sort but with them another

example similar in its essentials was repeatedly met. In urethanized individuals given a hypertonic solution and placed on the back the intestinal coils in which fluid accumulated out of the body soon raised the abdominal surface here and there, and over these slight protrusions the color of the stained surface tissues turned toward yellow.

Frequently in the crouching, depleted rabbit a slight, generalized change toward yellow, localized to the tissues of the lower part of the sides, preceded the characteristic patchy acidosis. The patching was never so pronouncedly orange-yellow as in rats.

Relation of Local Ischemia to the Acidosis.

An evident cause for the color changes over bony projections and low on the sides of rabbits is to be found in the existence of slight pressure differences,—differences devoid of effect under ordinary circumstances. We have already given reasons for the supposition that the ordinary patchy acidosis of depletion is referable to a lessened volume flow resulting from compensatory vasoconstriction. To learn how far the circulation was interfered with recourse was had to the injection of a second highly diffusible vital stain into animals in which a first one (phenol red) had already disclosed the acidosis.

Experiment VII. The Patchy Outlying Acidosis Is Due to Patchy Ischemia.—Rats and rabbits were stained and depleted in the ordinary way and then injected with a solution of purified brom phenol blue (Hynson, Westcott and Dunning). This indicator has a range too far to the acid side for its color to be influenced by the reaction of the tissues. We have found that a 4 per cent solution of it in water, brought to pH 7.4 with NaOH and rendered isotonic with the blood by the addition of salt solution,* is an excellent vital stain, producing no symptoms, and coloring the normal animal an even deep blue by diffusion into the tissues, within less than 5 minutes after its intravenous injection. 0.4 cc. suffices for the purpose in the case of a rat of 150 gm. and 3 cc. per kilo for rabbits. The staining of the tissues persists for many hours. In a succeeding paper the characters of the dye as a vital stain will be dealt with.

In normal animals colored with phenol red the introduction of brom phenol blue causes the hue of the body surface to change rapidly from red to a deep plum-purple; and the change takes place evenly. In animals with a patchy acidosis,

* 400 mg. of brom phenol blue is ground in an agate mortar with 40 mg. of sodium chloride and 1.2 cc. of N/1 sodium hydroxide. The mixture is brought to 10 cc. with distilled water and filtered.

however caused, the alteration is much more gradual and is highly irregular owing to the fact that the brom phenol blue fails entirely to enter the orange (acidotic) patches. These retain their hue whereas the red-stained tissue between them

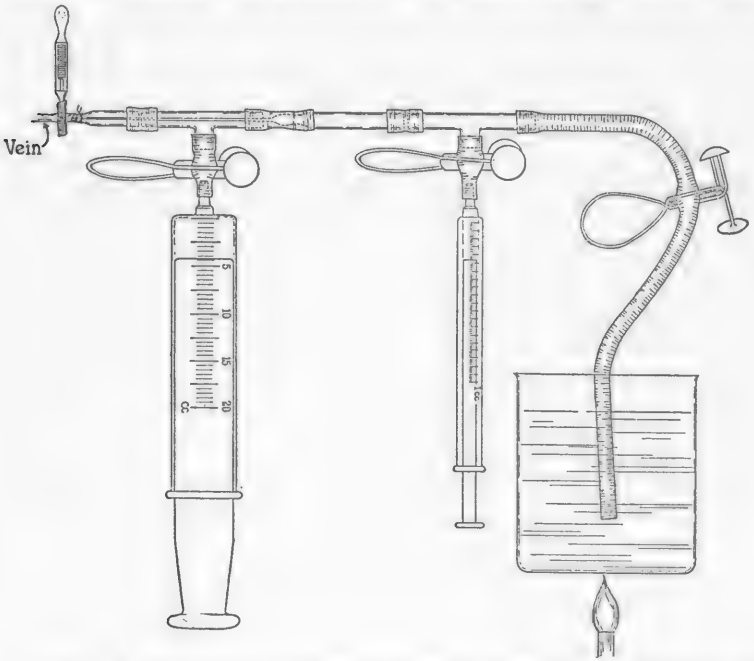

FIG. 1. Apparatus for the injection of a small quantity of warm solution into a tiny vein.

The injection apparatus is first brought to body temperature by drawing the warm dye solution through it by suction on the 20 cc. syringe. The solution passes from beaker to syringe by way of a capillary pipette extending inside the cannula nearly to the tip. When enough has been drawn through to warm the system the connections with beaker and 20 cc. syringe are rapidly clamped off and the injection is made by forcing the desired quantity of solution from the small syringe into the system, thus driving an equivalent amount of the warm fluid from this latter into the vein.

turns purple. Thus, within 10 minutes as a rule, the animal appears spotted all over with orange blotches on a plum-purple background, the blotches being separate or more or less confluent, according to the degree of the depletion. A later progression through the stage of generalized acidosis to that of death of the animal

is without the least effect on the particolored mottling. For with the increasing failure of the circulation the brom phenol blue remains stranded in the regions to which it had at first been distributed; and such secondary color changes as come about in the phenol red in response to the general acidosis do not suffice to alter the patterning. On the other hand in animals recovering from depletion the orange patches gradually merge with their surroundings and disappear as the blue dye still present in the blood enters them. In rats killed at the height of the patching its pattern is long maintained, being lost partially and little by little by diffusion of the blue dye.

On stripping the pelt it could be seen that the furred regions were affected equally with those that had been shaved. There was even a fine mottling of the ears and of the soles of the feet. In not a few cases phenol red was dispensed with. The depleted animals then appeared blotched with white on blue, and the control ones an even and deeper blue.

To test whether the superimposed brom phenol blue had any influence to determine the blotching sodium indigotate (the sodium salt of indigodisulfonic acid) was sometimes substituted for it. In order to color the animal deeply without introducing too much fluid a 2 per cent solution in water was used. The dye precipitates out at room temperature so a special cannula was devised to ensure that it was warm when it entered the femoral vein (Fig. 1). In depleted rats given no phenol red but injected with 1 cc. of indigotate the characteristic mottling, this time of white on indigo blue, was obtained. It proved possible to preserve the mottled pelts in acetone, after they had been rapidly removed and stretched.

The General Blood Pressure during the Local Ischemia.

Here is proof that the patchy outlying acidosis is associated with a failure of fluid exchange within the patches, a failure so entire that highly diffusible dyes cannot enter them. The ischemia responsible for the patching would seem to be as complete as that induced by epinephrin and comparable in this respect to epinephrin ischemia. The question arises of the way in which it comes about. Can one suppose it due in part to inherent local differences in the conditions making for interchange between blood and tissue? No signs of any such differences are to be seen in normal animals during the process of vital staining. The surface tissues color evenly. But it might well be that under the circumstances of a greatly diminished blood pressure local influences of no moment ordinarily would become effectual. An example of such a phenomenon has already been described in the influence of prominences beneath the skin to determine regions of local acidosis in depleted rabbits. To decide the matter observations were

made on depleted animals to find whether the acidotic patchiness develops while the general blood pressure is still well maintained. This proved to be the case.

Experiment VIII. The Blood Pressure in Animals with Outlying Acidosis.— To determine the blood pressure the carotid artery was entered in the manner already described, with a needle of rather large bore (No. 23), mounted upon the barrel of a tuberculin syringe which in turn was connected by means of a water column with a manometer of about 1 mm. bore containing mercury (Fig. 2). Just before the needle was introduced some three-fourths saturated $MgSO_4$ was drawn up into it to prevent clotting and the mercury column was adjusted to

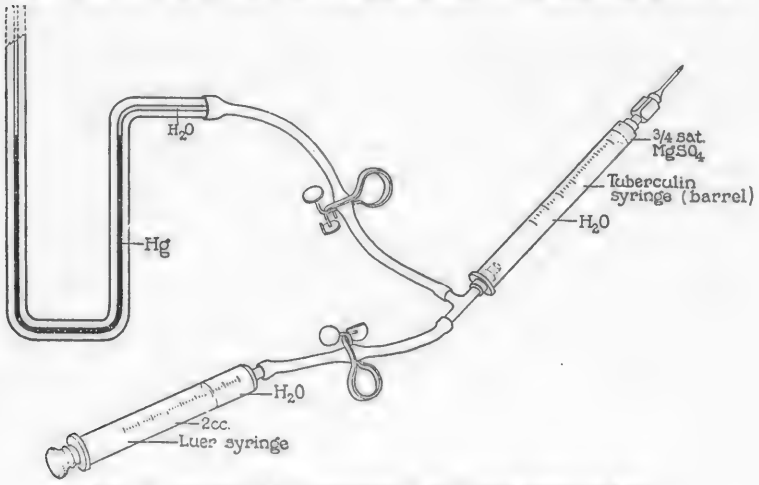

FIG. 2. Apparatus for determining the blood pressure of rats.

slightly less than the expected pressure. In this way the loss of blood was minimized. It could never have been more than a few hundredths of a cc.

The rats were urethanized, stained, and depleted by the administration of hypertonic sodium carbonate solution. A grave objection to the method was found in the progression of the anhydremic condition during the period in which local acidosis was developing to the point at which it became evident in a change of the surface color. Even when a local ischemia is induced with epinephrin the bloodless state endures for some minutes before sufficient acidosis develops to bring about a color change. It follows that under the circumstances of a progressive anhydremia the blood pressure prevailing when local acidosis becomes manifest must frequently be lower than at the moment when the local ischemia responsible for said acidosis comes into being.

The blood pressure in rats that were merely urethanized and stained ranged between 100 and 120, averaging about 105. In a fasting animal of 185 gm., given 3½ cc. of sodium carbonate, a characteristic patchy acidosis had developed after 20 minutes although the pressure was then 112 mm. Hg. 20 minutes later still the dose of carbonate was repeated, with result that after the same interval of time, or an hour in all, the spotting had become very pronounced. The pressure was now 67 mm. Hg. In a second rat of the same weight given only 3.5 cc. of carbonate a marked patchy acidosis was present after 20 minutes, and the blood pressure was found to be 91. In a third animal it was 88 after 25 minutes, when patchy acidosis had appeared. By the time a generalized acidosis had developed in these rats, and in others, some of which were depleted by bleeding, the pressure was lower, usually about 65 mm.

The carotid blood pressure of rats proved surprisingly high; and it was still well maintained after bleedings which sufficed to induce a patchy outlying acidosis. One is justified in the view that this last is referable to a patchy vasoconstriction, not to intrinsic local differences in the conditions determining interchange between blood and tissue.

The diffuse acidosis of the subcutaneous tissue observed as the result of large bleedings may be in part the result of a lessened oxidation traceable to the diminished number of red cells. But the relative unimportance of this factor is proved by the disappearance of the acidosis as the blood undergoes the dilution incident to restoration of its bulk.

Distribution of the Ischemic Patching.

Thus far we have confined ourselves to the happenings in the superficial tissues. But during the course of the work numerous evidences were obtained that an ischemia of the sort responsible for patchy acidosis of the subcutaneous regions occurs elsewhere. To study this ischemia proved easier than to study such acidosis as may have resulted from it. For the reaction of most of the organs is too far to the acid side for phenol red to be useful with them; and, as has already been stated, the color changes with brom cresol purple are frequently not clear-cut. One can readily tell, however, where local ischemia prevails by vital staining with brom phenol blue. That acidosis often results from the ischemia is a fair inference, even though it cannot always be disclosed by the indicators at present available.

We shall here confine ourselves to the phenomena occurring in animals depleted for the study of outlying acidosis. In later papers the distribution of the ischemia consequent on various disturbances will be dealt with at length.

Experiment IX. The Distribution of Ischemia in Depleted Animals.—Animals stained with phenol red were depleted by bleeding or by anhydremia induced with sodium carbonate. When a patchy acidosis of the superficial tissues had disclosed itself they were injected intravenously with 4 per cent brom phenol blue, and killed some minutes later by decapitation, the object being to remove as much as possible of the circulating dyes. The organs were inspected swiftly and the order in which they were looked at was varied from animal to animal.

At first urethanized rats and rabbits were used. After a considerable number of experiments had been performed the papers of Henning (17) and of Florey and Marvin (18) appeared, in which proof is brought that urethane interferes with some at least of the vascular responses. This rendered necessary a repetition of the work in the absence of a general anesthetic. Rats were depleted by aspiration from the heart and injected into a superficial leg vein. A special technic was devised in order to bleed the rabbits. Under ether anesthesia a common carotid was freed but not severed, and brought to the surface; a paraffined cannula was introduced into it, and the muscles were sewed together beneath in order that it should remain superficial. To keep it closed it was tied down with coarse silk upon a short piece of rubber tubing, the spring of the tubing against the soft ligature being sufficient to occlude the lumen. The incision was then closed loosely. When bleeding was to be done some hours later the tissues were moistened with novocaine as they were exposed, and the ligature was cut against the rubber. When the bleeding had been finished and a new ligature put on, the cannula was washed free of blood with salt solution. In this way the vessel could be opened and closed again and again, with the rabbit in a sitting position. It was found that the gradual removal within half an hour of somewhat less than half the total blood volume gave rise to a patchy change toward orange here and there on the body surface. The color differences were never so pronounced as in rats, and much more depletion was required to produce them. The rabbits exhibiting them still sat or moved about as normally. Warmed 4 per cent brom phenol blue was now injected into an ear vein and 3 minutes later the head was taken off at a blow. When experience had shown how much depletion was needed to induce local ischemia the staining with phenol red was frequently omitted.

The findings in the superficial tissues have already been described. As remarkable a state of affairs was encountered in the voluntary muscles. These color up evenly and rapidly with brom phenol blue under normal circumstances, and by the end of 3 minutes they are everywhere blue. But in bled or anhydremic rats, and in bled rabbits, staining takes place more slowly and according to a definite pattern, with many unstained regions regularly interspersed amongst the blue

ones. When phenol red has been omitted the long muscles appear transversely banded with blue. The bands are slightly zigzag, with the blue fading gradually at the edges. The mackerel sky appearance to which they give rise can be seen without dissection in the quadratus lumborum when the peritoneal cavity is opened. It is also visible in some muscles that are more or less flat, as in the pectorals and the gracilis. In sheet muscles such as the external and internal oblique there is, instead of the banding, a regular dappling of oval unstained areas on a blue background, or, when depletion has been great, a mere reticulation of blue on muscle tissue that is for the rest unstained. The amount of unstained tissue varies in other words with the degree of depletion. In rats injected with phenol red only, and then bled or rendered anhydremic the presence in the muscle of spots yellower than their surroundings, relatively acid that is to say, can with some difficulty be made out. A superimposed staining with brom phenol blue discloses the fact that this acidosis affects regions not entered by the blue dye. When depletion has been great the muscles stained with phenol red are orange-yellow throughout.

No study of the kidney was made. After many observations on the other large viscera we can say definitely that patchy ischemia fails to develop in them, even when the blood bulk has been so greatly diminished that death inevitably follows within a brief time. The spleen frequently appeared practically unstained with brom phenol blue, standing out vividly red against its dark colored surroundings. This evidence that the organ is shut off from the circulation in depleted animals is what might have been expected from the studies of Barcroft.

In the cartilage of the ears of depleted rabbits stained with phenol red patchy acidosis does not occur but there develops a generalized one, the tissue turning orange-yellow. It stains slowly but evenly with superimposed brom phenol blue. Acidosis was not to have been expected in the tendons since even mechanical interference with the circulation fails to cause it, for a long time at least; and none was found. Brom phenol blue failed to disclose any patchy ischemia of the bone in those few osseous regions into which the dye penetrates during the brief period after its injection.

These observations make plain that the contraction of the vascular bed which occurs in compensation for a diminished blood bulk affects the organs not only to different degrees,—a fact long known,—but in different ways. In the subcutaneous tissue and skeletal muscle of moderately depleted animals there occurs a well-ordered patchy ischemia so complete that the regions affected are to all intents and purposes no longer served by the blood. In consequence they become relatively acid. Lying between the acidotic areas are others in the same tissue which manifest the normal reaction and are by comparison excellently supplied with blood. The viscera show no patchy ischemia visible in the gross, but the entire spleen is often deprived of circulation for long periods.

DISCUSSION.

The direct proof that acidosis develops during life in tissues ill served by the blood merely substantiates a conclusion reached in the past on excellent indirect evidence. But such evidence has not enabled investigators to tell when local acidotic states will develop, much less anything about their distribution, intensity, and course. The method of vital staining with indicator dyes yields information on all these points.

Previous experiments (8, 14) have brought out the fact that changes induced in the reaction of the blood by the direct injection into it of acid or alkali, by breathing high percentages of carbon dioxide, or by hyperventilation are all closely accompanied by changes of essentially the same magnitude in the reaction of the subcutaneous tissue. In the present paper the existence is described of conditions in which a parallelism of this sort fails to obtain. Often a frank and not inconsiderable tissue acidosis develops, unassociated with any alteration in the blood reaction or with a slight one at most. It can even happen that the blood becomes more alkaline while the tissues are growing acid. Though the determining causes for these discordances are various, those that we have studied have a common physiological basis in circulatory shortcomings.

The observations on epinephrin ischemia have shown that where the circulation to the subcutaneous tissue of rats is interrupted a relative acidity develops within but a few minutes. When the blood volume of an otherwise normal animal is abruptly diminished by hemorrhage or by the administration of a hypertonic solution there develops a widely distributed tissue acidosis even though the general blood pressure is still well maintained and the blood pH unchanged. Such acidosis is traceable to the same cause as that due to epinephrin, namely vasoconstriction. This is not to say that the method whereby vasoconstriction is induced is necessarily devoid of any influence of its own. Animals depleted with hypertonic sugar solution show a more pronounced tissue acidosis than those given hypertonic sodium carbonate or bled; the acidosis tends to be more widely distributed throughout the subcutaneous tissue; it is more often accompanied by a lowering of the blood pH; and the acidotic tissue when exposed to

the air reverts toward alkalinity slowly and slightly. There exist numerous reports on the complex metabolic disturbances caused by introduction into the body of sodium carbonate or bicarbonate. The lactic acid of the blood is increased in quantity (19–21), and acetone bodies are voided in the urine (22). From the protocols of some of the work one can perceive that so much carbonate was given as must inevitably have led to anhydremia, local ischemia, anoxemia, and acidosis. For nearly 40 years it has been known that conditions of anoxemia result in a formation of lactic acid (23). But in the most recently described instances of carbonate ketosis, anhydremia can scarcely have played a rôle.

One would expect that in anhydremic animals the increased viscosity of the blood would act as an adjuvant to vasoconstriction and local ischemia would occur under circumstances of less reduction in blood volume than when hemorrhage is the effectual agent. We have gained the impression that this is so, without having proved it.

Outlying acidosis must develop in numerous pathological conditions of man which are attended by faulty interchange between the blood and tissues. The literature gives one every reason to suppose it a not infrequent complication of the clinical anhydremias. What is known of the physiology of shock, when viewed in the light of our findings, justifies the belief that the blood acidosis encountered in that state but feebly expresses the degree of acidosis existing in certain of the tissues. The same is true of the blood changes observed after severe hemorrhages. Hertzmann and Gesell (24) have noted that on suddenly restoring the blood volume after bleeding a temporary fall in the pH of the fluid may occur. The suggestion has been made (25, 26) that local acidotic accumulations may suddenly be swept into the blood with result in harm to the organism. Our observation, that the ventilation of tissues which have become acidotic as the result of simple ischemia brings about a rapid reversion in them toward alkalinity, would seem to indicate that the relatively innocuous carbon dioxide plays a major rôle in determining local acidity under such circumstances. In the failure of this reversion to occur in animals rendered anhydremic with sugar one may perceive evidence of the formation of non-volatile acid substances.

The question whether long continued local acidosis brings about

local pathological changes is not without clinical implications. In this connection some observations of the present work may be recalled, in which regions of local acidosis over knees and spine were determined in depleted animals by pressure differences of no consequence under normal circumstances. It seems possible that similar pressure differences, coming into play under pathological circumstances, might explain the localization of certain skin lesions seen clinically. But while the pressure factor may well be of importance here, local acidosis as such will scarcely suffice as a cause for the lesions themselves. The sedentary clerk sitting all day upon tissue that is inevitably ischemic and acidotic sustains no local injury from this cause. Connective tissue cells continue to live and proliferate after they have rendered the medium about them markedly acid (27), and they will withstand much larger alterations in reaction, produced with CO_2, than any attained in acidotic tissues (28, 29).

Many cells throughout the body normally contain vacuoles that are acid in reaction; and both polymorphonuclear and mononuclear phagocytes elaborate an acid material around particles that they engulf. Indicators of suitable range when taken up by such cells show the hue of acidity so long as these elements live, but that of alkalinity when, dying, they become permeable to the alkaline fluids round about (30). Such observations cause one to ask whether local tissue acidosis may not develop in the absence of circulatory derangements. The evidence we have gained by altering the blood pH through the introduction of acid or alkali, showing, as this did, rapid and precisely concomitant changes in the reaction of the matrix tissues, is all against any such happening in the intercellular substances. On the other hand enormous accumulations of relatively acid material may occur within cells, as is evident when they segregate litmus (30), the peritoneal coat, for example, then being much thickened and colored deep red by elements which have converted the indicator from the alkaline form and stored it. A segregated acidosis of sorts here surely exists as it must under other circumstances. Of the protoplasm itself nothing is known in this relation. The color of living parenchymal cells (liver, pancreas) stained with a phthalein indicator does not alter when enough acid or alkali is introduced into the organism to bring about pronounced changes in blood pH and the pH of the matrix tissues.

One would expect the speed with which outlying acidosis develops, and perhaps its intensity, to be less in rabbits than in rats, since the smaller the mammal the higher ordinarily is its metabolic rate. And this is the case. Rabbits stained with phenol red and depleted change color slowly as compared with rats, and the color attained is never so yellow. In the stained dog an ischemia produced with the tourniquet or with epinephrin results in an evident tissue acidosis only after the lapse of 15 minutes or more. In two dogs repeatedly bled under ether no patchy acidosis could be elicited, but a slight general one became evident after a considerable interval. On the other hand patchy ischemia with acidosis is readily induced in the cat by bleeding, as one of us, with Dr. H. P. Gilding, has recently found. The time required for the development of the acidosis seems to be much the same as in the rabbit. A young white pig which had shown pronouncedly the reactive hyperemia of Bier (31), and the stroking phenomena intensively studied by Lewis (32) was injected with phenol red and bled under ether in the attempt to bring out a superficial patching. None occurred, nor did the skin as a whole change color though the depletion was pushed so far that the animal died. Not until nearly an hour after death was a slight change in the body hue noted, such as would accompany a fall of pH 0.1. It follows that a patchy ischemia may have been present during life without causing sufficient local acidosis for it to have become evident through a color change. The animal was very fat.

Clinical reports on cutaneous phenomena, when viewed in connection with our findings, give one every reason to suppose that a local ischemia with acidosis occurs not infrequently in man. Unfortunately no indicator is as yet available for utilization in this connection. The pale orange hue assumed by weak solutions of phenol red injected directly into superficial acidotic areas is too frequently masked by the skin color to be generally helpful.

The patchy ischemia disclosed by staining methods in depleted animals is one expression of a widespread and well-ordered circulatory readjustment obtaining under emergency conditions. It seems not to have been recognized before. A further report upon it will be published shortly.

SUMMARY.

In various functional conditions involving peripheral vasoconstriction a more or less widespread change toward acidity takes place within certain tissues. The change is frequently independent of any in the blood. Indeed the blood can become more alkaline while the tissue acidosis is developing.

When the blood volume is diminished abruptly but not too greatly, by hemorrhage or by anhydremia, the acidosis which develops in the superficial connective tissue and in the skeletal muscles is patchy in distribution, being limited to areas of local ischemia themselves the result of a compensatory vasoconstriction which affects certain regions only. There is a second type of patchy ischemia (and of acidosis) which occurs under circumstances of moderate depletion and is referable to local pressure differences that are so slight as to be ineffective under normal circumstances. A generalized acidosis throughout the superficial tissue develops when depletion is extreme. All these are outlying acidoses, since they lie without the influence of the blood. In the viscera no such acidoses have been found.

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THE SEPARATION OF TYPES AMONG THE PNEUMOCOCCI HITHERTO CALLED GROUP IV AND THE DEVELOPMENT OF THERAPEUTIC ANTISERUMS FOR THESE TYPES.*

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In connection with the study of the use of concentrated refined antibody preparations in the treatment of pneumonia due to Types I, II and III it seemed desirable to study the possibility of treating pneumonia cases due to pneumococci of the miscellaneous types hitherto classified as Group IV. In general, clinical results have indicated that these cases are benefited very little by treatment with the therapeutic antibody preparations now in use. Furthermore, since some cases due to these organisms are severe and a considerable percentage develop septicemia it seems worth while to try to find a suitable serum therapy for them.

The classification of pneumococci into Types I, II and III and Group IV is generally known and accepted. Other types have been differentiated, but the grouping has not been generally utilized, probably due to the absence of diagnostic or therapeutic serum suitable for use with the new types and to the belief that the types did not occur in lobar pneumonia with sufficient frequency to warrant attention. Lister (1) found another type more prevalent than Types I, II and III in South Africa. Olmstead (2) in a study of 213 strains by direct agglutination found that the miscellaneous strains which she studied could be classified in twelve groups. Clough (3) classified 94 strains in twelve groups. Strains giving delayed or incomplete but marked agglutination with Type II serum were classified by Avery (4) as Subgroup II strains and separated by agglu-

* This investigation was aided by grants of money from the New York University Littauer Pneumonia Fund and the Metropolitan Life Insurance Influenza Commission Fund for part of the expense.

tination and protection tests into Subgroup II A and Subgroup II B. Stillman (5) described further Subgroup II strains on the basis of agglutination tests. Among 55 strains isolated during the influenza epidemic, we (6) differentiated nineteen types which represented four small groups and thirteen unrelated strains. Griffith, (7) studying forty-nine strains not Types I, II or III from respiratory diseases, thirty-four of which were from lobar pneumonia, distinguished twelve serological types. He found that strains corresponding to the Sub II A and Sub II B of Avery predominated. Robinson (8) found eight groups among 65 strains of the so called Group IV. One group was identified as the cause of an epidemic. Sugg, Gaspari, Fleming and Neill, (9) and Harris, Sugg and Neill (10) described a group of strains which were immunologically related to Type III.

In the present investigation it was necessary, preliminary to the selection of suitable strains for the production of therapeutic serum, again to find into what types the miscellaneous strains were divisible and which types were most prevalent. The majority of the strains[1] were isolated at the Harlem Hospital; a smaller number[2] from the Bellevue Hospital. 120 strains from lobar pneumonia cases which did not agglutinate or reacted atypically with diagnostic antiserums for Types I, II and III, were studied. Strains of Subgroups II A and II B were obtained from Dr. Avery at the beginning of the investigation; and later, in order to learn something concerning the distribution of the types, strains were obtained from Dr. Robinson representative of the types found in Pittsburg, also a strain of a non-typical Type III from Dr. Sugg of Nashville, Tennessee. Antiserums for five of the types found in England were sent us by Dr. Griffith. Monovalent antiserums were prepared and agglutination tests carried out. By direct agglutination tests, the 120 case strains studied were divided into ten groups containing four or more strains each, one group of only two strains, and two strains differing from each other and from the preceding types, and a miscellaneous group differing from all the preceding types and among which further types were not separable with the antiserums thus far prepared. It is apparent that additional agglutinating antiserums might divide the group of undifferentiated strains thus adding to the number of types. The search for new types is to be continued. We are designating the ten types having

[1] We are indebted to Miss Dedrick, Miss Brown and Miss Gideon for these cultures.

[2] We are indebted to Dr. Sutliff for these cultures.

four or more strains by Roman numerals from Type IV to Type XIII. The agglutination results with representative strains of each type and antiserums prepared for each type are given in Table I. Only a small amount of cross-reaction occurred among the types. When rabbit antiserums are employed this is usually the case; generally there is more cross-reaction when horse antiserums are used.

Each group of strains was studied in regard to the ability of representative strains to stimulate the production of protective antibodies.

TABLE I.

The Agglutination Titer of the Antiserums for Representative Strains of Each of the Recently Separated Types with the Homologous Strains and Strains of Each of the Other Types.

Type strains	Type antiserums									
	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
IV	1–640	1–5	—*	1–20	—	1–20	—	—	—	—
V	1–10	1–320	—	1–20	—	—	1–5	—	—	—
VI	—*	1–40	1–160	—	—	—	—	—	—	—
VII	—	1–20	—	1–640	—	1–5	1–20	—	—	—
VIII	—	—	—	1–40	1–640	1–20	—	—	—	—
IX	—	1–5	1–5	1–20	—	1–640	1–5	—	—	—
X	—	—	—	1–20	—	—	1–640	—	—	—
XI	—	—	—	—	1–40	1–20	—	1–320	—	—
XII	—	—	—	—	—	1–5	—	—	1–160	—
XIII	—	—	—	—	—	1–5	1–5	—	—	1–160

* Indicates that no agglutination was found in dilutions as low as 1–5.

The power of antiserums produced by injection of a single strain to protect against freshly isolated as well as old strains belonging to the same type, was determined. The amount of cross-protection of antiserums for one type against strains of other types was studied also. Antiserums for at least two strains of each type were prepared. Cultural or morphological peculiarities of the strains were noted. The special data are given in detail in a discussion of each group of strains.

Type IV.—Twenty-one strains are of this type, nineteen were from recent cases and two were stock strains. One of the stock strains is an "antitoxin" strain from the Lilly Laboratories, the other a repre-

sentative of one of the groups, "Group IV B," obtained from Robinson. Of the nineteen cases due to this type, sixteen were rated[3] as severe and seven of these died; three were rated as mild. Four of the patients who died and one who recovered had positive blood cultures.

The strains of Type IV had a peculiarly high virulence for mice, all being fully virulent when first tested and remaining so throughout the

TABLE II.

Protection Tests Showing the Comparative Protection of a Polyvalent Type I, II and III Concentrated Antibody Preparation and of an Antiserum for Type IV against a Type IV Culture.

Polyvalent Types I, II and III antibody preparation		Monovalent Type IV antiserum (rabbit)	
Antiserum amounts	Results in mice	Antiserum amounts	Results in mice
cc.		*cc.*	
0.1	+48 hrs.	0.001	Survived
0.05	+24 hrs.	0.0005	Survived
0.02	+48 hrs.	0.0002	Survived
0.01	+24 hrs.	0.0001	+48 hrs.
0.005	+48 hrs.	0.00005	+48 hrs.
		0.00002	+30 hrs.
	Amount	Colony count	Results in mice
	cc.		
Control	0.000001	153	+48 hrs.
of virulence	0.0000001	16	+48 hrs.
of culture	0.00000001	2	+48 hrs.

Method: 0.001 cc. of culture (0.5 cc. of dilution 1–500 in broth) and the desired amount of serum diluted with saline (0.5 cc. of required dilution) was drawn into a syringe and injected intraperitoneally into mice.

Control: 0.5 cc. of the dilution made in broth containing the required amount of culture and 0.5 cc. broth was drawn into a syringe and injected into mice.

The approximate number of diplococci injected were estimated by colony count of blood agar poured plates.

period of the investigation. By fully virulent we mean that ten or fewer diplococci killed mice in less than 48 hours. This dose was usually contained in 0.00000001 cc. of 18 hour broth culture as shown by colony count in poured blood agar plates. In order to test the

[3] We are indebted to Dr. Bullowa and Dr. Rosenbluth for the ratings made in the clinical investigation of pneumonia at the Harlem Hospital and to Dr. Sutliff for those at the Bellevue Hospital.

potency of antibody stimulated by injection of this type, an antiserum from a rabbit inoculated for 5 weeks with a representative strain was tested to determine its protective value against the strain injected and fifteen other strains classified in this group. The results indicated 100 to 500 protective units[4] against each strain. The remaining strains were not tested.

The protective value of a trivalent Type I, II and III concentrated antibody preparation having 1500 protective units against Type I, 1000 against Type II and 20 against Type III was tested against Type IV. This preparation had less than one unit per cc. of protection for Type IV, whereas the control homologous antiserum (rabbit) had 500 units of protection per cc. The results of these tests are shown in Table II. Monovalent antibody preparations for Type I, for Type II and for Type III and other trivalent antibody preparations than the one mentioned above were tried with similar results. It is apparent that the antibody preparations for Types I, II and III are practically useless for treatment of Type IV infections and that antibody preparations to be efficient must be homologous to this type. With this in view the immunization of horses with a Type IV strain was begun. After 21 weeks of injections the antiserum from a horse had 100 protective units per cc. being suitable for diagnostic and experimental therapeutic use.

It is interesting that our Type IV corresponds with one of the types, "Pn. 10," found by Griffith in England. His strains were isolated from three cases of lobar pneumonia, one of bronchopneumonia and one of acute bronchitis. Our Type IV also corresponds with the "Group IV B" differentiated by Robinson in Pittsburg which included six strains from lobar pneumonia with a case mortality of 33 per cent. Considering the limited number of cases in both series the mortality figure closely approaches ours of 37 per cent. Because of its apparent wide distribution and its virulence it is expected that this type will prove to be of especial importance.

Type V.—Six strains from cases are included in this group and two stock strains, the Subgroup II A strain of Avery and a strain of Group IV E of Robinson. All the pneumonia cases due to this type were

[4] A unit is ten times the smallest amount of serum which protects mice against 100,000 fatal doses of a fully virulent culture inoculated intraperitoneally.

especially severe. Five out of six of the patients were shown to have positive blood cultures and these cases died.

All the strains examined of this group when cultivated in blood broth media showed a tendency to hemolyze blood cells; with some strains the tendency was very much more marked than with others

TABLE III.

Protection Tests Showing the Comparative Degree of Protection of a Type II Concentrated Antibody Preparation and of a Type V Antiserum against a Type V and a Type II Strain.

Protection against Type V				Protection against Type II			
Type II antibody		Monovolent Type V antiserum (rabbit)		Type II antibody		Monovalent Type V antiserum (rabbit)	
Antiserum amounts*	Results in mice	Antiserum amounts	Results in mice	Antiserum amounts	Results in mice	Antiserum amounts	Results in mice
cc.		*cc.*		*cc.*		*cc.*	
0.02	Survived	0.002	Survived	0.0002	Survived	0.1	+22 hrs.
0.01	Survived	0.001	Survived	0.0001	Survived	0.05	+46 hrs.
0.005	+96 hrs.	0.0005	+48 hrs.	0.00005	Survived	0.02	+46 hrs.
0.002	+30 hrs.	0.0002	+48 hrs.	0.00002	+72 hrs.	0.01	+52 hrs.
0.001	+48 hrs.	0.0001	+48 hrs.	0.00001	+48 hrs.	0.005	+46 hrs.
0.0005	+48 hrs.					0.002	+22 hrs.
	Amount	Colony count	Results in mice		Amount	Colony count	Results in mice
	cc.				*cc.*		
Control	0.000001	144	+48 hrs.	Control	0.000001	120	+48 hrs.
of virulence	0.0000001	12	+48 hrs.	of virulence	0.0000001	12	+48 hrs.
of culture	0.00000001	2	+48 hrs.	of culture	0.00000001	3	+48 hrs.

* 0.001 cc. of 18 hour broth culture was injected with the different amounts of antiserum.

Method: See foot-notes to Table II.

but in general it was greater than that of the other pneumococcus cultures studied. This ability to produce hemolytic substances may account for the severity of the septicemic cases. The strains when first isolated from patients were all highly virulent for mice, but did not hold their virulence as well as the strains of Type IV. After the virulence had fallen attempts to raise the virulence sufficiently high to carry out accurate protection tests were usually unsuccessful.

As noted above the Subgroup II A of Avery is of this type. Avery found that the strains were agglutinated to a considerable degree in Type II serum although the agglutination was usually delayed and often incomplete. He also found that Type II antiserum protected to some extent against Subgroup II A cultures while antiserum for Subgroup II A failed to protect against Type II cultures. Our results have been similar to his.

In order to find whether the therapeutic preparations, now available, were potent against infection with this type, protection tests were carried out using polyvalent antibody preparations for Types I, II and III, and monovalent antibody preparations for Type I, for Type II and for Type III. The Type II antibody preparation having 2000 units against Type II had only 10 to 20 units per cc. against the Type V strain. The polyvalent antibody preparations also had 10 to 20 protective units per cc. against Type V strains. Type I and Type III antibody had less than 1 unit per cc. against Type V. Comparative tests with the serum of a rabbit immunized against a Type V strain and with Type II antibody are shown in Table III. The rabbit serum for the Type V strain had 100 units of protection per cc. It is evident that a preparation containing 10 to 20 units per cc. would be of little value as compared with one containing 100 units per cc. or more. Neither the polyvalent antibody preparations for Types I, II and III nor the Type II antiserum is suitable for therapeutic use against infections with this type. Since diagnosis and treatment must be carried out independently of Type II we have thought it better to give the type an entirely distinct designation to avoid confusion. Potent antiserum for determining the type and for therapeutic use has been obtained by inoculation of horses.

Griffith found this type second in prevalence among the types into which he separated Group IV. Robinson found this type, termed by him "Group IV E," most numerous during the winter of 1928 in Pittsburg.[5] On account of its prevalence, virulence and special tendency to infect the blood stream, this type should be of especial interest for serum treatment.

Type VI.—Seven strains from pneumonia cases are included in this

[5] Personal communication.

group, also one stock strain, the Subgroup II B strain of Avery. Of the seven patients two had positive blood cultures and died. Three of the cases were rated as moderate in severity and two as mild. The case strains of this type were generally of somewhat lower virulence for mice than those of Types IV and V. Those which were virulent when isolated had a tendency to lose their virulence very rapidly. Type VI strains hemolyzed blood less actively than Type V but more actively than the majority of the other pneumococcus cultures studied. The strains of Type VI are similar to these termed Sub II B by Avery. He found the relationship of this type similar to that of the Subgroup II A strains to Type II. He found that Subgroup II B was immunologically distinct from Subgroup II A. Our results were similar. We found that monovalent or polyvalent preparations which were highly protective against Type II would protect against Type VI to a slightly greater degree than against Type V. However, protection to a high degree was obtained with antiserums prepared by injections of Type VI strains. The largest group separated by Griffith from the so called Group IV corresponded with our Type VI. He found it next in prevalence to Types I and II.

The data available indicate that infections with this type are milder than with Types IV or V and it may be expected that cases will respond more readily to treatment.

Type VII.—Eleven strains from cases of pneumonia were of this type. Seven cases were rated as severe and two of these died. Two cases were moderate in severity. No data were available in regard to the ratings of the other two cases. One of the fatal cases and one case which recovered had positive blood cultures.

The strains of Type VII were generally of low virulence for mice. None were suitable as test strains for carrying out protection tests as the virulence could not be raised. Therefore the protective value of a therapeutic antiserum (horse) prepared for this type could not be accurately determined by the mouse protection method. Possibly another method may be devised. This antiserum is suitable, however, for determining the presence of this type.

Type VIII.—Four strains from cases and two stock strains are of this type. Of the stock strains one is a representative of the "Group IV A" of Robinson and the other is the "Thomas" strain of Sugg.

Of the four cases three were rated as severe and one as moderate. One patient who had a positive blood culture died.

The Type VIII strains were found to agglutinate in Type III antiserum to a marked degree but less than typical Type III strains,

TABLE IV.

Protection Tests Showing the Comparative Degree of Protection of Monovalent Antiserums for Type III and Type VIII against a Type III and a Type VIII Strain.

Protection against Type VIII				Protection against Type III			
Type III antiserum (rabbit)		Type VIII antiserum (rabbit)		Type III antiserum (rabbit)		Type VIII antiserum (rabbit)	
Antiserum amounts*	Results in mice	Antiserum amounts	Results in mice	Antiserum amounts	Results in mice	Antiserum amounts	Results in mice
cc.		cc.		cc.		cc.	
0.1	+27 hrs.	0.001	Survived	0.002	Survived	0.1	Survived
0.05	+22 hrs.	0.0005	Survived	0.001	Survived	0.05	Survived
0.02	+22 hrs.	0.0002	+78 hrs.	0.0005	+45 hrs.	0.02	Survived
0.01	+27 hrs.	0.0001	+45 hrs.	0.0002	+45 hrs.	0.01	+45 hrs.
0.005	+45 hrs.	0.00005	+22 hrs.	0.0001	+27 hrs.	0.005	+45 hrs.
0.002	+27 hrs.					0.002	+23 hrs.
	Amount	Colony count	Results in mice		Amount	Colony count	Results in mice
	cc.				cc.		
Control of virulence of culture	0.000001	214	+27 hrs.	Control of virulence of culture	0.000001	163	+48 hrs.
	0.0000001	34	+45 hrs.		0.0000001	24	+48 hrs.
	0.00000001	1	+72 hrs.		0.00000001	1	Survived

* 0.001 cc. of 18 hour broth culture was injected with the different amounts of antiserum.

Method: See foot-notes to Table II.

this would lead to confusion of the more reactive of these strains with Type III in routine "typing" tests. Two strains of Type VIII were sent us from a hospital in response to our request for freshly isolated Type III cultures. It is impossible, at present, to judge the prevalence of this type, as other strains belonging to it may have been classified as Type III in the routine hospital tests. We were receiving the

strains which apparently did not fall with Types I, II and III. We are planning to study all the presumed Type III strains isolated during the coming season and will then have more definite information on this point.

Protection tests were carried out with monovalent rabbit antiserum for Type VIII and for Type III. The results with the monovalent rabbit antiserums are given in Table IV. The antiserum for the Type VIII strain had 500 protective units per cc. against Type VIII strains (four strains tested), but only 20 units per cc. against a stock Type III strain and 1 unit against a freshly isolated Type III strain. An antiserum for a stock Type III strain had 100 protective units per cc. against the stock Type III strain, 25 units against a freshly isolated Type III strain and less than 1 unit per cc. against Type VIII strains. The most potent Type III concentrated antibody available had only 1 to 2 protective units or less against Type VIII strains. Polyvalent antibody for Types I, II and III failed to protect.

A very interesting and detailed study of this type has been published recently by Sugg, Gaspari, Fleming and Neill, and by Harris, Sugg and Neill. Our findings agree in general with theirs. The "Group IV A" of Robinson corresponding to this type, was one of the three types of miscellaneous pneumococci found most prevalent in Pittsburg during the winter of 1927. The mortality among his cases was 14 per cent.

The differentiation of this type from Type III is very important in the investigation of the use of antibody preparations in Type III cases. At least some part, even if small, of the failure with Type III antibody treatment is probably due to the failure to exclude cases due to this type. We hope that in the future this type may be identified and we are preparing a specific antiserum for therapy.

Type IX.—Six strains from pneumonia cases are of this type. One of the cases was rated as severe, four as moderate, one as mild. One patient who had a positive blood culture died. All strains were of comparatively low virulence for mice when tested. Perhaps this was because the strains had been grown for some time on artificial media before the classification was made and virulence tests were carried out. Now that an antiserum for identification of this type is available it is hoped that more virulent strains suitable for protection tests will be

encountered. An antiserum has been secured for this type by the inoculation of a horse.

Type X.—Five strains from cases of pneumonia are of this type. All were from severe cases, three were fatal, two of which were shown to have positive blood cultures. The cultures were of moderate or low virulence for mice. An homologous rabbit antiserum had 500 protective units per cc. while a polyvalent antibody preparation for

TABLE V.

Grouping of Pneumococci of So Called Group IV.

Types	Total strains including stock strains*	No. of strains from cases 1927–1928	Percentage of case strains in each type	No. of deaths	Percentage of deaths in each type	No. of cases having positive blood cultures
Type IV	21	19	15.8	7	36.8	5
Type V	8	6	5.0	5	83.3	5
Type VI	8	7	5.8	2	28.6	2
Type VII	11	11	9.2	2	18.2	2
Type VIII	6	4	3.3	1	25.0	1
Type IX	6	6	5.0	1	16.7	1
Type X	5	5	4.2	3	60.0	2
Type XI	4	4	3.3	1	25.0	1
Type XII	4	4	3.3	2	50.0	2
Type XIII	4	4	3.3	0	0	0
Total strains typed	77	70	58.3	24	34.3	21
Strains not typed	50	50	41.7	11	22.0	15
Total strains	127	120	100.0	35	29.2	36

* Stock strains obtained from Dr. Avery, of The Rockefeller Institute for Medical Research, Dr. Robinson, of the William H. Singer Memorial Research Laboratory, Pittsburg, and Dr. John Y. Sugg, of the Vanderbilt University, Nashville, Tennessee.

Types I, II and III had only 1 unit against Type X. The results indicate that in therapy this type also must be considered as an entity.

Type XI.—Four strains from cases of pneumonia are of this type. One patient died. Three cases were rated as moderate in severity and one of these had a positive blood culture. Type XI strains have a moderate tendency to hemolyze blood. They were of moderate to high virulence for mice when first isolated and lost their virulence rapidly.

A rabbit immunized with a representative of the type had 200 to 500 protective units per cc. against the different strains classified as this type. A polyvalent antibody preparation for Types I, II and III had 1 to 5 protective units or less against Type XI strains.

Type XII.—Four strains from cases of pneumonia are of this type. Three of the four cases were rated as severe and were shown to have positive blood cultures. Two of the cases were fatal. The fourth case was moderately ill. Type XII strains had moderate virulence for mice.

Type XIII.—Four strains from cases of pneumonia are of this type. One case was rated as severe, three as moderate. None of the cases were fatal. These strains were of moderate virulence for mice when first isolated but rapidly lost their virulence.

The total number of strains studied, including the stock strains, the number from lobar pneumonia cases, the deaths with their percentages and the number of positive blood cultures are given in Table V. Since the number of cases studied is comparatively small, the figures may be deceptive as to the actual prevalence of the types and the mortality of the cases. This is especially true in regard to the mortality; as the infecting organism would be more easily isolated in pure culture from severe cases and would be grouped, while in the lightly infected cases it might not be recovered.

Monovalent antiserums for each of the types were tested to find whether there was any protective power against representative strains of each of the other groups where strains of sufficient virulence were available. These tests were made with the serum from immunized rabbits and very little protection was found, *i.e.*, less than 1 unit per cc. against heterologous strains. Concentrated Type II antibody preparations and concentrated polyvalent antibody preparations having a high potency against Type II were found to have 10 to 20 units against Types V and VI; an amount too low to ensure efficient treatment. These results indicate that to be effective a serum should contain antibodies stimulated by a type similar to the infecting type. Protection experiments have shown that monovalent antiserums of high protective value as determined by laboratory tests can be prepared for most of these types by inoculation of rabbits and horses and it is hoped that this will be true for the remaining types where at

present the work is not sufficiently advanced to make a definite statement. The final estimate of their value must be determined by the clinical use in the treatment of actual pneumonia cases.

In a recent paper Park, Bullowa and Rosenbluth (11) with reference to the unfavorable results of serum treatment of Type III cases, discussed the difference in the ability of Type III antiserums to protect against freshly isolated Type III strains as compared with old stock strains. The almost complete failure of the antiserums to successfully combat freshly isolated strains is probably due both to the low potency of the antiserum and to the remarkably large capsules which these organisms develop. The stock strains have smaller capsules, although they are "fully virulent" according to the definition which we have given above. The Type III stock strains had a much greater antibody stimulating power than the freshly isolated strains. Thus an antiserum prepared with a stock strain had 500 protective units against the stock Type III strains and 25 or less protective units against freshly isolated Type III strains, while an antiserum prepared with a freshly isolated strain had 5 units against the stock strain and considerably less than 1 unit against the freshly isolated strains. None of the freshly isolated strains of Types IV to XIII equalled Type III in the development of capsules, although there was considerable difference among representative strains in regard to this tendency. All surpassed freshly isolated Type III strains in antibody stimulating power. From these observations it is deduced that the new types are of a nature which should be susceptible to the action of suitable antiserums.

It is evident that the preparation of so many different type antiserums and the determination of the infecting type and the carrying out of specific treatment, while possible, will be a very laborious procedure. It would be very much simplified, if suitable polyvalent antiserums could be prepared, which would be effective against a large or considerable number of types and if a polyvalent antiserum could be used for identification of cases suitable for treatment. We are studying the possibility of preparing such a therapeutic antiserum. Certain difficulties have already been encountered, in as much as, the potency for the separate types is lowered somewhat in proportion to the number of strains injected. Possibly by combining the refined

and concentrated antiserums of horses immunized each against a few strains, a preparation can be obtained which will be sufficiently potent.

SUMMARY.

The pneumococci hitherto known as Group IV have been separated into ten types which have been designated by Roman numerals from IV to XIII. These have been correlated as far as possible with the types described by others. The prevalence and mortality of cases due to each type have been estimated in the limited number of cases studied.

Laboratory tests indicated that therapeutic antiserums for Types I, II and III have very little protective power against the recently separated types.

Monovalent antiserums of high agglutinative and protective power were prepared in rabbits for each type.

Several monovalent antiserums each specific for a type, which are suitable for agglutination and experimental therapeutic use, have been obtained by immunizing horses.

An antiserum prepared for one type had very little cross-protective power against other types.

A study of the possibility of preparing a suitable refined and concentrated polyvalent antiserum has been begun.

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STUDIES ON BACTERIAL ENZYMES.

VII. Lactase and Lipase of the Colon Bacillus.

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In this paper proof will be brought that lactase and lipase enzymes are endocellular constituents of the colon bacillus. While the colon bacillus is only on occasion a pathogenic organism, it assumes importance as a normal inhabitant of the intestines. Knowledge of the mechanism by which it attacks lactose is particularly desirable because the fermentation of this sugar with production of acid and gas is the basis of its differentiation from the typhoid bacillus and other bacteria causing intestinal diseases. In the present investigation lactase and lipase were demonstrated by the lactose-splitting and tributyrin-splitting properties of sterile solutions of the intracellular substances of the bacilli.

EXPERIMENTAL.

Methods.

Preparation of Colon Bacillus Enzyme Solutions.—The enzyme solution was prepared from typical colon bacilli. Essentially the same procedure was employed as in the previously described preparation of enzyme solutions from other bacteria (1–4).

A concentrated suspension of the bacterial cells was obtained from the surface growth of agar cultures; the bacteria were washed and resuspended in salt solution. The suspension was distributed into 15 × 200 mm. Pyrex tubes and subjected to repeated freezing and thawing as previously described (1–4). After about 250 repetitions of the freezing process, microscopical examination showed that the majority of the cells had been at least partially disintegrated. The material was then centrifuged at high speed and the supernatant fluid filtered through a Berkefeld filter.

The final sterile solution was water-clear, but contained a considerable amount of dissolved bacterial substance as indicated by its coagulation when boiled. This

solution of the dissolved intracellular substance, entirely devoid of formed cells or fragments, served as the enzyme solution.

Sterility Controls.—After filtration, the sterility of the enzyme solution itself was proved by cultures in glucose broth. No antiseptics were added to the enzyme-substrate mixtures; the sterility of each was controlled by cultural methods.

Lactose-Hydrolyzing Property of Sterile Solutions of Colon Bacterial Substances.

The principal object of the experiment was to determine whether or not the sterile solution of the intracellular substances of colon bacilli possessed the property of hydrolyzing lactose. Since living cultures of the colon bacillus produce acid and gas from lactose, tests were also made to determine whether or not the sterile bacterial solution possessed the property of formation of acid or of gas either from lactose or from the hexoses yielded by its hydrolysis.

TABLE I.

Action upon Lactose of Sterile Solutions of the Intracellular Substances of Colon Bacilli.

Test mixture	Lactose hydrolysis	Action on hexose products
	Changes in pH due to dysentery bacillus fermentation of hexoses previously formed by colon bacillus enzyme	Acid or gas production
	ΔpH	
Lactose plus active (unheated) bacterial solution....	1.4	0
Lactose plus heat-inactivated bacterial solution.....	0.0	0

Since lactose is a reducing sugar, its hydrolysis was tested by a biological method (described in detail in preceding papers (1, 2)), which is based upon the acid fermentation of hexoses by bacteria which do not ferment the particular disaccharide from which the hexoses are derived. 1.0 cc. of each of the test and control hydrolysis mixtures was put into a sterile Pyrex tube; 1.0 cc. of a suspension of non-lactose-fermenting dysentery bacilli was then added to the different tubes. These mixtures (equal portions of the original enzyme-substrate mixtures plus the bacterial fermenting agent) were incubated at 38°C. for 1 hour after which the bacteria were removed by centrifugation. The production of acid was detected by colorimetric

pH estimations of the clear supernatant fluids. Sufficient controls were included to limit all changes in reaction of the fermentation test mixtures to acid produced by the dysentery bacillus fermentation of hexoses previously split from the lactose by the active colon bacillus enzymes.

The original enzyme-substrate mixtures were sealed with vaseline during their incubation and tested for acid or gas production immediately before the tests for lactose hydrolysis.

The results are summarized in Table I.

The results of these tests showed that the sterile bacterial solution possessed the property of hydrolyzing lactose but was devoid of the property of forming gas or acid either from lactose itself or from the hexoses yielded by the disaccharide hydrolysis. These data present experimental evidence of the possession by the colon bacillus of a heat-labile endocellular lactose enzyme. The capacity of the sterile solution of intracellular substances to hydrolyze lactose and its lack of capacity to produce acid or gas from the hexose products furnish another example of the distinction previously observed between the properties of sterile bacterial solutions and the properties of living cultures of the same bacteria (1–6). The capacity of acid and gas fermentation is apparently more intimately dependent upon the morphological integrity of the bacterial cell, for of the two reactions (disaccharide hydrolysis and hexose fermentation) involved in the action of the living colon bacillus upon lactose, only the hydrolytic activity remains operative in the sterile filtered solution of the intracellular substances.

Splitting of Tributyrin by Sterile Solutions of Intracellular Substances of Colon Bacilli.

Tests of the glyceride-splitting property of the sterile bacterial solution were made with tributyrin suspended in phosphate solution. As shown in Table II, the fat was hydrolyzed with acid production by the unheated bacterial solution and not by the heat-inactivated control. This presents experimental evidence of the possession by the colon bacillus of a heat-labile lipase. Since the bacterial solution contained only intracellular substances derived from washed bacilli, the lipase of the colon bacillus is an endocellular constituent of these bacteria. In this respect it agrees with the lipases of pneumococci (7)

and Welch (3) and anthrax* bacilli, but differs from the lipase of the *botulinus* bacillus which is an extracellular substance (2).

DISCUSSION.

This paper reported a demonstration of the presence of an active lactase and an active lipase (tributyrinase) enzyme in sterile, filtered solutions of the intracellular substances derived from physically disintegrated colon bacilli. The lactose-splitting and tributyrin-splitting action operated in the entire absence of formed cells or cell fragments. These enzymes represent heat-labile endocellular constituents of the colon bacillus.

TABLE II.

Splitting of Tributyrin by Sterile Solutions of Colon Bacilli.

Test mixture	Change in reaction due to splitting of tributyrin
	ΔpH
Tributyrin plus active (unheated) bacterial solution	1.5
Tributyrin plus heat-inactivated bacterial solution	0
Phosphate solution plus active, or plus heat-inactivated bacterial solution	0
Salt solution plus tributyrin solution	0

The demonstration of the lactase contributes to the knowledge of the mechanism of lactose fermentation which is one of the most important biochemical activities of colon bacilli. It is commonly assumed that hydrolysis to hexoses is the first step in the fermentation of disaccharides by bacteria. Evidence that this is true for a number of different bacteria has been furnished by demonstrations of the presence in sterile bacterial solutions of the active hydrolyzing enzymes specific to the same disaccharides that are fermented by the living organisms (1–6). However, there is no evidence that it is true for all species of bacteria, and in fact a number of authorities (7) have concluded that certain kinds of bacteria can attack complex sugars without their preliminary hydrolysis. It is desirable, therefore, to

* Unpublished experiments in this laboratory have proved that anthrax bacilli possess a heat-labile endocellular fat-splitting enzyme.

obtain direct proof of the hydrolyzing enzymes rather than to assume their possession by all bacteria that produce acid from the respective disaccharide. Since the colon bacillus is the classical representative of the "acid-gas" group of sugar-fermenting bacteria, it is of particular interest to obtain direct evidence that it possesses an active endocellular lactase. The fermentation of lactose is the basis of the distinction of colon bacilli from the pathogenic bacteria of intestinal infections and the lactase enzyme acquires importance as being responsible for the first step in this process.

SUMMARY.

Colon bacilli possess endocellular heat-labile lactase and lipase enzymes which remain operative in sterile filtered solutions of the intracellular substances obtained through physical disintegration of the bacillary bodies. The demonstration of the lactase and detection of the hexose products of its action constitute experimental evidence that hydrolysis of the disaccharide is the first step in the fermentation of lactose by colon bacilli.

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THE NATURE OF THE HYPERGLYCEMIA ASSOCIATED WITH ANAPHYLACTIC SHOCK IN THE DOG.

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The experiments reported in this paper were carried out with the purpose of gaining further data on the factors which are involved in the release of glycogen from the liver. Anaphylactic shock was used as a means of radically altering the blood sugar level and producing a condition in which glycogenolysis is known to occur.

The hyperglycemia resulting from the injection into rabbits of killed bacteria of various types (Menten and Manning (1), Zeckwer and Goodell (2), Levine and Kolars (3)) was found to be prevented by removing one adrenal and cutting the splanchnic nerve of the opposite side (Evans and Zeckwer (4)), and in such animals the bacterial injection caused often a pronounced fall in blood sugar, sometimes to a convulsive level. It was thought that anaphylactic hyperglycemia, in which the curve is quite similar to that of bacterial hyperglycemia, might be altered by a similar surgical procedure, and could be more completely studied in dogs.

Several studies have been made of carbohydrate metabolism in anaphylaxis. O'Neill, Moy, and Manwaring (5) found a marked disappearance of liver glycogen during canine anaphylactic shock. The occurrence of hyperglycemia associated with anaphylactic shock has been reported in the guinea pig (Zunz and La Barre (6)), in the dog (McCullough and O'Neill (7), Achard and Feuillé (8)), and in rabbits (Zeckwer and Goodell (9)). The mechanism of this hyperglycemia has recently been studied, chiefly in the guinea pig by La Barre (10), and his results will be discussed later.

Contradictory data on whether the adrenals discharge adrenalin in anaphylactic shock have been given by Houssay and Molinelli (11), who report a slight discharge of adrenal secretion in dogs as determined by the denervated heart method; by Smith and Ravitz (12), who found no change in the epinephrin content of the

adrenals, by chemical determination, during anaphylactic shock in guinea pigs and rabbits; and by La Barre (10), who anastomosed the adrenal vein of one dog to the jugular vein of a second dog, and reports that when anaphylaxis was evoked in the first dog there was no adrenalin effect in the second dog.

The frequency of contradictory results and interpretations by different workers studying the mechanism of hyperglycemia under various experimental conditions is probably due to the fact that the blood sugar level is a peculiarly delicate balance between opposing forces, so that in attempting to study the rôle of a given factor it is almost impossible to avoid complicating the picture by other factors which may vitiate the results. There are, however, certain conditions which should be eliminated from all studies of carbohydrate metabolism which frequently are not. Anesthetics such as ether or chloroform upset so obviously the carbohydrate metabolism that it is difficult to draw any conclusions from experiments carried out under them. This error has entered unavoidably into much of the work before 1923, when amytal was found by Page (13) to be an anesthetic which has little effect on the blood sugar level. In many experiments, drastic operative procedures have been performed immediately preceding the experiment proper. The trauma and shock of such an operation leads to unavoidable discharge of liver glycogen and circulatory changes which complicate the picture and lead to incorrect conclusions.

The present experiments were designed to avoid all unnecessary complicating conditions. Amytal was used as the anesthetic which in the hands of many workers, and in our own previous experience, has permitted the continuance of a low steady blood sugar level, which does not interfere with the production of experimental hyperglycemia and hypoglycemia. In those animals in which adrenalectomy and splanchnic section were carried out, the operation was performed several weeks antecedent to the acute experiment, so that at the time when shock was induced the animals were in vigorous health.

The present experiments were limited to the phenomenon of anaphylaxis as seen in the dog. In the guinea pig, where bronchial spasm is the outstanding feature, an asphyxial hyperglycemia is to be expected. Likewise in the rabbit, the asphyxial element undoubtedly enters into the condition. In the cat, the unsensitized animal reacts so strongly to serum, that a toxicity quite apart from anaphylaxis obscures the picture. In the dog, however, the phenomenon is one fundamentally of profound fall in blood pressure, with intense engorement of liver and intestine. There is no important respiratory disturbance in the anesthetized anaphylactic dog according to Pearce

and Eisenbrey (14), and no pulmonary vascular phenomena according to Drinker and Bronfenbrenner (15). The dog then would seem to be a good animal in which to study the release of glycogen uncomplicated by asphyxia.

In canine anaphylaxis there is evidence that the liver cell is directly affected. Weil (16) found that when antigen is injected directly into a portion of the liver in a sensitized dog, that portion of the liver becomes intensely congested. Numerous observations by Manwaring and his collaborators (17) indicate the essential part the liver plays in canine anaphylaxis. The lessened coagulability of the blood and the liver's reduced capacity to form urea from ammonium lactate (Rumpf (18)) also point to the involvement of the liver cell in the process. It is conceivable then that without the intervention of the adrenals the liver cell might be stimulated directly to give up its glycogen under the circumstances of anaphylaxis by some such mechanism as change of pH in venous stasis, or by direct toxic effect.

Experimental Procedure.

Dogs were divided into two groups, (1) normal intact animals, and (2) those which had been operated upon, under ether anesthesia, through the lumbar route on each side, the left adrenal being excised and the right splanchnic nerve being transected beneath the diaphragm, and usually a piece of nerve trunk being removed. Sensitization was begun after recovery from the operation.

Both groups of dogs were sensitized according to the method used by Manwaring, Marino, McCleave, and Boone (19), being given about 0.5 cc. horse serum per kilo body weight subcutaneously followed on the next day by the same amount intravenously. Shock was produced from 24 to 43 days later.

Just before the acute experiment, the animals were fasted 14 hours. They were then anesthetized with amytal in alkaline solution given intraperitoneally in doses of 50 to 80 mg. per kilo body weight. Enough N/2 NaOH was added to the amytal to put it into solution. With the first twelve dogs, amytal in tablet form was used, and this required more alkali for solution than the amytal in powdered form used in all subsequent experiments, which required about 8.8 cc. of the alkali per 1 gm. of amytal. The dogs varied greatly in their susceptibility to amytal. In many, the calculated dose was given and did not lead to complete anesthesia, so that additional doses, with corresponding amount of alkali, had to be given.

The animals were placed on a warmed table; one carotid artery cannulated for blood pressure tracings; a tracheal tube inserted for artificial respiration, which was used routinely to offset any respiratory disturbance which might arise during shock; one femoral artery prepared for taking blood for blood sugar analyses, and one femoral vein incannulated for the injection of serum. A number of blood sugar determinations were made during the preparation of the animal and

over a period of about an hour after the minor operative procedures had been completed and before the serum was injected, in order to have evidence that the blood sugar level was quite steady at the time when anaphylaxis was induced. A blood pressure tracing was secured usually before the artificial respiration was turned on, as a standard to insure that there was no change in blood pressure resulting from the artificial respiration. Another control of hyperventilation or hypoventilation was given by the series of blood sugar analyses after the artificial respiration was instituted and before anaphylactic shock had been induced.

To produce anaphylactic shock, serum was introduced very slowly into the femoral vein until the blood pressure began to fall. From 0.17 to 0.81 cc. serum was used per kilo body weight. Immediately after injecting the serum, blood samples were taken every few minutes for the first half hour, thereafter at less frequent intervals.

The blood samples were oxalated, the proteins immediately precipitated, and the filtrates allowed to accumulate until the whole series was finished. The Hagedorn and Jensen (20) method for blood sugar was used because it requires only 0.1 cc. blood for each analysis, and permits of running a large number of filtrates at the same time. This method gives slightly lower values than the Folin and Wu method, but relative not absolute values sufficed for the purpose of this investigation.

At the end of each experiment in the operated animals, the removal of the left adrenal and the denervation of the right was verified by dissection. In seven animals the glycogen content of the liver was determined at death in order to demonstrate the presence of available glycogen. In four dogs the oxygen content of the arterial blood was determined before and during shock.

RESULTS.

A typical experiment is given in Table I.

The chief results are given in Table II and Fig. 1. A control for the effect of serum administration and the effect of the experimental procedure on the blood sugar in the absence of anaphylaxis was given by Dog 1 (operated) and Dog 21 (unoperated).

Two experiments (Dogs 4 and 5) were carried out to repeat the curves of hyperglycemia in anaphylaxis obtained by McCullough and O'Neill (7). These two experiments resulted in hyperglycemia but of a lower degree, as McCullough and O'Neill had used ether and had consequently a high level of blood sugar to begin with.

Having thus checked the effective but non-fatal dose of serum and the proper time intervals, and having demonstrated the occurrence of hyperglycemia under the given experimental conditions, anaphylactic shock was then induced in a series of animals, in which one

TABLE I.

Showing a Typical Experiment, with Marked Rise of Blood Sugar in Anaphylactic Shock of Unoperated Dog in Presence of Adequate Ventilation.

Time		Blood sugar	Oxygen content of arterial blood
		per cent	*Volume per cent*
5/14/28	Dog 26, male, weight 11 kg.		
5/15/28	4.4 cc. horse serum injected subcutaneously		
5/16/28	4 cc. horse serum injected intravenously		
6/27/28	Weight 11.5 kg.		
a.m.			
9.45	Blood from femoral vein for blood sugar. Amytal 0.700 gm. given intraperitoneally, 60 mg. per kg. body weight	0.082	
10.00	Partial anesthesia		
10.10	Well under anesthesia. Strapped on heated table. Right carotid incannulated		
10.17	Tracheal tube inserted but not connected with artificial respiration apparatus. Anesthesia deep		
10.40	Blood pressure recorded. Blood pressure 140 mm. Hg.		
10.52	Blood from femoral artery for blood sugar	0.107	
11.06	Blood from femoral artery	0.103	
11.15	Blood pressure being recorded. Artificial respiration turned on		
11.23	Blood from femoral artery. Rectal temperature 38°C.	0.098	
11.48	Blood from femoral artery. Blood pressure 140 mm. Hg.	0.100	
p.m.			
12.15	Blood from femoral artery, collected under oil	0.100	17.62
12.25	Began injecting serum slowly into femoral vein		
12.25½	Blood pressure beginning to fall. Injection stopped		
12.28	Blood pressure 30 mm., has fallen 110 mm. Hg below previous level. Absence of the usual accessory respiratory movements	0.087	
12.34	Blood from femoral artery	0.087	
12.40	Blood from femoral artery, collected under oil	0.177	18.90
12.47	Blood pressure beginning to rise, 50 mm. Hg		
12.52	Blood from femoral artery. Blood pressure rising. 80 mm. Hg	0.171	
1.00	Accessory respiratory movements. Blood from femoral artery	0.145	
1.06	Blood pressure has returned to about normal level		
1.08	Blood from femoral artery	0.120	
1.15	Blood from femoral artery	0.092	
1.30	Blood from femoral artery	0.073	
1.50	Blood from femoral artery	0.073	
2.20	Blood. Liver glycogen 1.6 per cent Animal killed	0.084	

adrenal had been removed and the opposite splanchnic nerve cut (Dogs 3, 6, 11, 12, 13, 15). There was a slight rise in blood sugar, which occurred usually within 5 minutes, reached its maximum in 7 to 15 minutes, and returned to normal in about 30 minutes. In no

TABL

		Controls		Anaphyla			
Animal	Dog	1	21	4	5	17	20
	Weight in kg.	7.3	11.4	7.4	9.1	11.6	10
	Sex	F.	M.	M.	M.	F.	
Time of experiment	No. days after operation	42					
	No. days after sensitization			24	30	43	24
Dose of amytal	Total gm.	0.550	0.700	0.600	0.550	0.830	0.7
	Gm. per kg. body weight	0.074	0.061	0.081	0.061	0.071	0.0
Blood pressure after injection of serum	Time after beginning of injection when B.P. reached lowest. Min.			3¼	3	1½	2¼
	Fall in mm. Hg. below previous level	0	0	70	106	96	116
	Min. after injection when B.P. began to to rise			9	4	18	6
	Min. after injection when B.P. returned approximately to normal			35	8	60	20
Blood sugar	Minimum and maximum values before injection. Gm. per 100 cc. blood	0.063	0.103	0.090	0.101	0.064	0.0
		0.075	0.115	0.123	0.112	0.071	0.1
	Average of preliminary values	0.067	0.111	0.101	0.103	0.067	0.1
	Maximum value after injection	0.061	0.101	0.188	0.163	0.090	0.1
	Total gm. increase over average preliminary value	−0.006	−0.010	0.087	0.060	0.023	0.0
				Mean of series is 0.057 ±			
	Time after injection of highest B.S. value. Min.			35	60	28	16
	Time after injection of return to preliminary level. Min.			80		60	90
Liver glycogen per cent at end of experiment			2.4				

* Return of blood pressure incomplete.

† Figures refer to events after ligation of hepatic vein instead of after injection of serum.

instance was there a fall such as occurred in rabbits with adrenals inactivated, after bacterial injections (4). The preliminary level of blood sugar was low in the surgically treated animals. The same low level had been found previously to occur in rabbits submitted to the same operation (4).

In most of the dogs there was noticeable soon after the beginning of shock, alteration in the respiration. This consisted of spontaneous deep respiratory movements with increase in rate, in spite of the fact that abundant oxygen was being supplied to them by artificial respira-

II.

in unoperated dogs					Anaphylaxis after left adrenalectomy and section of right splanchnic nerve						Temporary constriction of hepatic veins	
25	26	28	29	30	3	6	11	12	13	15	21	31
10.2	11.5	12.5	12	10	7.5	9.0	11.3	9.7	7.8	9.4	11.4	15.0
M.	M.	M.	F.	M.	M.	F.	M.	M.	M.	M.	M.	M.
					42	27	33	49	60	38		
36	43	39	28	34	24	25	28	27	28	32		
0.627	0.700	0.750	0.780	0.750	0.500	0.700	0.800	0.750	0.550	0.480	0.700	0.900
0.061	0.060	0.060	0.065	0.075	0.066	0.077	0.071	0.076	0.070	0.051	0.061	0.060
5½	3	3½	4	5	2½	2	1¾	5	2½	3	1½†	3†
80	110	80	116	116	112	94	90	80	90	86	70	60
6	22	4½	8	17	Death	18	7	8	9	5½	12†	8†
40	41	6	30	60	*	*	14	40	*	13	20†	40†
0.091	0.082	0.105	0.110	0.077	0.061	0.055	0.074	0.062	0.064	0.067	0.103	0.096
0.122	0.100	0.109	0.127	0.131	0.081	0.077	0.080	0.078	0.084	0.076	0.115†	0.109†
0.107	0.098	0.106	0.115	0.108	0.072	0.068	0.078	0.069	0.071	0.071	0.111	0.103
0.129	0.177	0.114	0.217	0.177	0.075	0.081	0.106	0.121	0.094	0.109	0.212†	0.197†
0.022	0.079	0.008	0.102	0.069	0.003	0.013	0.028	0.052	0.023	0.038	0.101	0.094
0.006					Mean of series is 0.026 ± 0.004							
14	15	12	12	27		15	10	15	15	7	19†	10†
60	50	20	53			50	30	45	30	13	60†	60†
+	1.6	3.3			1.0					+		

tion. Spontaneous respiratory movements, however, could not be depended upon, for with Dogs 3 and 7, in which artificial respiration was not carried out, death occurred at the beginning of shock. Every attempt was made to ensure adequate ventilation, but when the experiments on the operated animals were completed, it was thought

that the greater increase in the blood sugar in the unoperated dogs might have been due to hypoventilation, especially in Dog 4, which had shown a temporary rise in blood sugar before the injection of serum had been made. Accordingly, anaphylaxis was induced in other unoperated sensitized dogs (Dogs 17, 20, 25, 26, 28, 29, 30). The rate and depth of the artificial respiration was set so as to secure roughly the maximum ventilation without causing hyperventilation with its associated fall in blood pressure. The adequacy of the ventilation was checked in Dogs 25, 26, 28, 30 by determining the

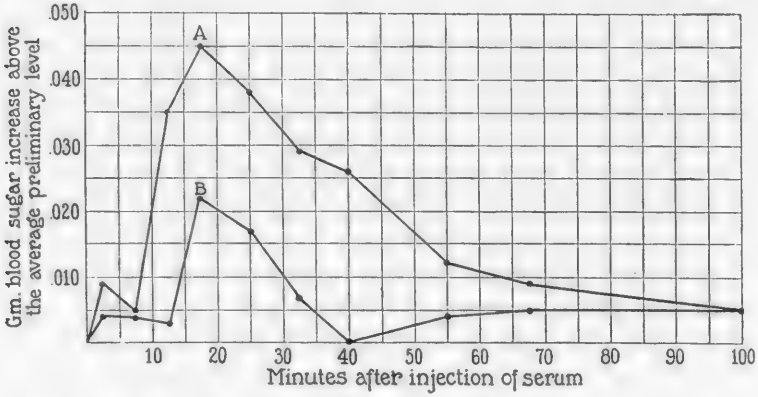

FIG. 1. Increase in blood sugar over preliminary level during anaphylaxis.
A = average of nine intact sensitized dogs.
B = average of six sensitized dogs in which one adrenal had been removed and the opposite splanchnic nerve cut.

oxygen content of the arterial blood withdrawn under oil before shock and at the height of shock (see Table III).

It was found that in two of these animals (Dogs 25 and 28), there was a slight decrease in the oxygen content during shock, as compared with that before shock, but these two dogs showed the least rise in blood sugar of the entire series of unoperated dogs, while in Dogs 26 and 30 which had shown a marked rise in blood sugar, there was a slight increase in oxygen content. These slight fluctuations in oxygen content were within the normal limits and probably represent minor alterations in the experimental conditions. These results seem to rule out asphyxia as a basis for the rise in blood sugar, as what altera-

tion occurred was in the opposite direction. However, since there is the possibility of slight change in the ratio of red cells to serum during shock, and such a change was found to occur in one dog in which the oxygen capacity of the blood when saturated with air was determined, it was thought that the determination of the percentage unsaturation of the blood would be a more accurate index of adequate ventilation than simply the oxygen content. Accordingly, in Dog 30, the oxygen content and the oxygen capacity when saturated with air were determined, and from these figures calculations of the percentage unsaturation were made before and during shock. The results (Table III) indicate a low percentage of unsaturation with no increase during shock. The hyperglycemia, then, associated with anaphylaxis is definitely not dependent upon hypoventilation.

TABLE III.

Showing Adequacy of Ventilation of Test Animals.

	Dog 25	Dog 26	Dog 28	Dog 30
Oxygen content, volume per cent				
Before anaphylaxis	19.89	17.62	19.09	16.95
After anaphylaxis	19.08	18.90	18.80	17.18
Oxygen capacity, volume per cent				
Before anaphylaxis				17.80
After anaphylaxis				17.80
Percentage unsaturation				
Before anaphylaxis				6.5
After anaphylaxis				5.2

In tabulating the results comparison was made between the operated and unoperated dogs, calculating the results both as percentage increase and as gm. blood sugar increase over the average preliminary level. It was realized that to express the change in the sugar content of the blood resulting from anaphylactic shock, in terms of percentage increase of blood sugar above the preliminary level does not give a fair picture of the change, as the lower preliminary blood sugar level commonly found in the operated animals would result in a relatively higher percentage increase than in the intact animals who showed a similar rise in gm. of blood sugar. Expressing the change in terms of the difference in gm. between the average preliminary level and the

highest level gave the fairest measure of comparison, and is the method of expressing the difference used in Table II.

From the results it is seen that all unoperated dogs showed some rise in blood sugar and six out of nine showed greater than 60 mg. increase, with a maximum of 102 mg. The operated dogs also showed a rise but of lesser degree, the maximum being 52 mg. One unoperated dog (Dog 17) differed from the others in this group, in that the preliminary level of blood sugar, for some unknown reason, was very low, like that of the operated animals. On analysis of these results, the mean percentage increase in blood sugar during anaphylaxis over the average preliminary level was 54.8 ± 5.9 per cent in the unoperated, and 36.6 ± 6.2 per cent in the operated, with a difference of 18.2 ± 8.5 per cent. Or expressing the comparison in terms of gm. blood sugar increase, which seems the preferable method of comparison, the mean increase in blood sugar during anaphylaxis over the preliminary level was 0.057 ± 0.006 gm. for the unoperated, and 0.026 ± 0.004 gm. for the operated dogs, with a difference of 0.031 ± 0.007 gm. However, the number of observations is too few to warrant precise mathematical analysis, and the problem did not justify carrying out additional experiments. In these experiments there are naturally wide deviations, but the operated animals tended to be more uniform in their reaction. The complexity of the uncontrollable physiological factors, such as degree of sensitization of the animal, amount of available liver glycogen, and individual variability in the activity of the autonomic nervous system, makes it possible to form only a rough estimate of the effect of any one factor such as operation.

DISCUSSION.

Effect of Operation.—The results then show a slight difference between the operated and unoperated animals. Cutting the splanchnic nerves obviously deprives of their innervation other structures than the adrenals, chiefly the liver, as far as carbohydrate metabolism is concerned. That the denervation of the liver, even when completely done, by stripping off the sympathetic fibres which course along the duodenohepatic artery, has little effect on the mobilization of glycogen such as by emotional stimuli, has been shown by Britton (21). Cutting the splanchnics appears to affect carbohydrate metabolism chiefly by cutting off impulse over them to the adrenal glands.

In our experiments the operation of removing one adrenal and cutting the opposite splanchnic nerve decreased the hyperglycemic reaction, but did not abolish it. The operated animals were in good health, had not lost weight, and the liver glycogen was abundant. The effect, therefore, was not due to any obvious fault in the metabolism of the animal. Probably sympathetic stimulation directly associated with anaphylactic shock is responsible for discharge of adrenalin and consequent glycogenolysis. The question then arises at to why, if the adrenals are concerned in anaphylactic hyperglycemia, there is still a positive, though relatively slight increase in blood sugar in the operated dogs. Were the adrenals only partly inactivated, or is there another factor in the production of the hyperglycemia? As far as could be determined by postmortem dissection, the operations had been complete. The same technique when employed by one of us had resulted in abolishing the effect of the adrenals in rabbits (4), but it may be that cutting the splanchnic nerve does not so completely denervate the adrenal in the dog as in the rabbit and cat. After these experiments were finished, publication was made of more elaborate operations for inactivating the adrenals (Izquierdo and Cannon (22), Zwemer and Newton (23)). Even if the operation as performed in the present experiments does not completely remove the activity of the adrenals the fact that there was a decrease in the blood sugar curves gave evidence that the operation had been at least partly effective, and that at least the adrenals were factors in the hyperglycemia. Furthermore the low preliminary blood sugar level gave evidence of the adrenals not being active. This being so, there must be a second factor concerned in the rise.

Effect of Mechanically Produced Venous Stasis of the Liver.

The question was considered whether purely mechanical venous stasis of the liver could release glycogen, and thus be responsible for the rise in blood sugar in the operated animals. In the dog, muscular bundles are particularly well developed in the hepatic veins, and Simonds (24) suggests that just as in the guinea pig where smooth muscle is particularly prominent in the bronchioles the manifestations of anaphylaxis are chiefly bronchial spasm, just so in the dog the engorgement of the liver can be accounted for as the effect of spasm of

the hepatic veins, and he found that in canine anaphylaxis there is very slight flow of blood from the hepatic veins. Whether contraction of the hepatic vein really contributes to the engorgement of the liver or whether the stasis of blood in the liver is entirely due to capillary dilatation in the splanchnic area, at least the condition of the liver is a conspicuous feature of canine anaphylaxis.

With Simonds' view in mind, the effect on the blood sugar level of temporarily constricting the hepatic veins was tried.

Preliminary experiments were carried out to develop a technique by which the venous outflow through the hepatic veins could be temporarily ligated without constricting the vena cava. Our technique differed from that of Simonds. In our experiments, a tape or narrow rubber tube was passed from right to left through the foramen of Winslow behind the hepatic artery and portal vein and bile ducts. This was then carried upward and backward between the liver and the diaphragm, then anteriorly across to the right between liver and diaphragm, then downward in the fissure dividing the right lobe of the liver from the main portion. The two ends of the ligature could then be drawn tightly together, and intense venous engorgement of the main portion of the liver occurred, without constricting the vena cava or obstructing the arterial supply to the liver. The right lobe of the liver was thus excluded from the constriction. This was found necessary, for if a ligature was thrown around the whole liver, the vena cava was definitely constricted. This ligation could be carried out rapidly with little trauma to the liver.

In Dog 21 this procedure was carried out. After a series of preliminary blood sugar determinations were made, the ligature was put in place but left loose. Two blood sugar determinations following this showed that the manipulation had had no appreciable effect on releasing liver glycogen. The tape was then tightly constricted. The main portion of the liver became blue and tense and the blood pressure fell to a low level. Blood samples were taken at short intervals. The ligature was held for 12 minutes, in order to simulate on the blood pressure record the effect usually obtained in anaphylaxis. On release of the ligature, the blood pressure promptly rose to normal, and the blood sugar rose to a high level, with subsequent fall. A similar experiment was carried out on Dog 31, constricting the liver 8 minutes, with resulting hyperglycemia (Table II). At the end of the experiment, histological sections were made of the liver, and no microscopic evidence of injury to the liver cells was found to have resulted from the constriction. In fact, a section from the portion of the liver

that had been constricted was indistinguishable from one taken from the unconstricted right lobe. The degree of hyperglycemia resulting from mechanical constriction was much higher than in anaphylaxis, as the mechanical occlusion of the hepatic vein is an exaggerated reproduction of the venous stasis occurring spontaneously in anaphylaxis. Further confirmation of the effect of venous stasis was thought unnecessary, as when these experiments had been completed, an investigation was published by Simonds and Brandes (25), in which they obtained a similar hyperglycemia on obstructing the hepatic vein.

The only previous study of the mechanism of hyperglycemia in anaphylaxis has been by La Barre (1927) (10), who found that hyperglycemia occurred in adrenalectomized guinea pigs, and although he states that the arterial blood became blue, he believes that asphyxia showed a much later effect on the blood sugar, and was not responsible for the immediate rise. He concluded that it was due to peripheral vagal stimulation because it occurred after vagal section and was not prevented by ergotamine. Since in the guinea pig asphyxia is the predominating feature of anaphylaxis, and the rapidity of the rise in blood sugar in asphyxia is well known, it does not seem convincing to rule out this factor. He did not use artificial respiration, and his operative procedures were done at the time of the acute experiment, which introduces many complicating factors. His view that vagal stimulation results in glycogenolysis is contrary to the opinion of most investigators who believe that the sympathetic, not the vagal fibres, are responsible for the release of glycogen.

From our experiments we conclude that in anaphylaxis as manifested in the dog, there are probably two factors responsible for the hyperglycemia, (1) sympathetic stimulation by way of the splanchnic nerves, involving the activity of the adrenals, and (2) increased glycogenolysis resulting directly from venous stasis of the liver.

SUMMARY AND CONCLUSIONS.

1. Nine unoperated dogs showed a rise of blood sugar during anaphylactic shock. In six of these dogs the rise was 60 mg. or over.

2. Six dogs in which one adrenal had long previously been extirpated and the opposite splanchnic nerve cut, showed a low preliminary level

of blood sugar, and a relative rise of blood sugar during anaphylaxis, but of less degree than in the unoperated animals. In no case was it greater than 52 mg.

3. Anoxemia did not appear to be a complicating factor, as evidenced by determination of the oxygen content of the arterial blood before and during shock.

4. The rise in blood sugar, which occurs in spite of the loss of adrenal activity, is probably due to the venous stasis of the liver seen in anaphylaxis in the dog, because this rise in blood sugar can be simulated in a normal non-sensitized dog by mechanically constricting the hepatic veins for a brief interval.

5. There are, therefore, probably two factors responsible for the hyperglycemia associated with anaphylaxis in the dog, sympathetic stimulation by way of the splanchnic nerves involving the activity of the adrenals, and glycogenolysis resulting directly from venous stasis of the liver.

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THE TOXICITY OF HUMAN SERUM FOR THE GUINEA PIG.

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The toxicity of normal human serum for the guinea pig has usually been referred to in the literature on the toxicity of heterologous sera without definite data on the variables to be met with in such studies. Thus De Kruif[1] states that normal human serum killed small guinea pigs in doses of 0.5 to 0.75 cc. but does not indicate the age of the serum. The inference from his experiments on the toxicity of normal rabbit blood for the guinea pig is that the effect is anaphylotoxin-like in nature, incident to clotting and certain experimental procedures. Taniguchi[2] reported that some "normal" human sera showed a toxicity for the guinea pig, and attributed this to their heterophilic antibody content (which he demonstrated definitely by suitable absorption tests) on the basis of the quantitative relationship between the primary toxicity of a given serum and its content in heterophilic antibody. On this ground, he proposed the hypothesis that the occasional anaphylaxis-like reaction in the human following the administration of horse serum was heterophilic in nature, since horse serum is known to contain a heterophilic antigen. But no actual study of the effect of such treatment with horse serum upon the primary toxicity of the human serum for the guinea pig has been found in the literature.

An increased toxicity was noted by one of us in a study of passive transfer of the anaphylactic antibody in the serum of certain cases of serum sickness.[3] The increased content in heterophilic antibody of such sera, as manifested in their capacity to hemolize and to agglutinate sheep cells, after differential absorption with rabbit and

[1] De Kruif, P. H., *J. Infect. Dis.*, 1917, xx, 717.

[2] Taniguchi, T., *J. Path. and Bact.*, 1922, xxv, 77.

[3] Tuft, L., and Ramsdell, S. G., *J. Immunol.*, in press.

guinea pig kidney emulsions, was definitely established.[4] (The same case numbers have been used in both studies.)

It is the purpose of this paper to report a preliminary study of the occurrence and nature of the effects of administering to guinea pigs human serum from 14 cases without history of serum treatment, 5 cases with history of such treatment without serum sickness, and 14 cases with history of serum sickness; in all of which the important factors of the age of the serum, its dosage, the size of the experimental animal, and the time relation of the bleeding of the patient to administration of the antiserum have been considered.

Technic.

The great variability in the reactivity of the guinea pig was found to depend on two factors: the weight of the animal and the dosage of the human serum. Following a number of experiments, optimal conditions were obtained by the use of animals of about 180 gm. and a uniform dosage of 1 cc. of the serum. This was given by a jugular puncture, without narcosis and with care to avoid loss of blood. That none of the ensuing symptoms of toxicity could be ascribed to physiological upset due to the volume of the injection was shown by the fact that a dose as high as 4 cc. of inactivated normal human serum was borne without the slightest sign of discomfort.

1. The Toxicity of Normal Human Serum.

Early in the study it became evident that every fresh human serum possessed a considerable toxicity for the guinea pig under the given experimental conditions. Typical results of a study of the duration of this phenomenon are shown in Table I. In no case was serum from a case without history of serum treatment found to be fatally toxic after 3 days of aging. It thus becomes possible to separate definitely the toxicity inherent in all fresh human sera from that found in aged serum after treatment of the patient with any of the usual antisera.

But it is to be noted that the response of the animal in the first case cannot be clearly differentiated from what will be shown to occur in

[4] Davidsohn, I., *J. Immunol.*, in press.

the second: the reactions are markedly similar, in both cases, to true anaphylaxis. The only clear differences between them lay in two manifestations found following the administration of fresh serum but not that of toxic aged serum. First, the frequent early appearance of a great weakness, especially in the hind quarters, which was not a paralysis, since the animal could move readily if provoked; second, in the absence at all times of froth from the mouth and nose. If the

TABLE I.

Showing the Effect of Time on the Toxicity of Fresh Human Serum.

Case No.	Age of serum	Dosage	Guinea pig reaction
		cc.	
45	5 hrs.	1	Prostration in 2 min. Death in 10 min.
		0.7	Convulsions in 2 min. Prostration in 3 min. Death in 20 min.
	6 hrs.	0.4	Jerky breathing in 10 min. Weakness. Recovery.
	4 days	2	No immediate effect. Occasional convulsive effort at 40 min. Recovery
54*	24 hrs.	1	Weakness in 2 min. Clonic seizures in 6 min. Prostration and death in 17 min.
	48 hrs.	1	Weakness in 2 min. Clonic seizures in 3 min. Prostration in 6 min. Death in 14 min.
	72 hrs.	1	1. Jerky breathing in 8 min. Few slight clonic seizures. Continued jerky breathing. Recovery 2. Slight clonic seizures in 5 min., lasting about 1 min. Jerky breathing. Recovery
56	24 hrs.	1	Clonic seizures in 3 min. Death in 5 min.
	48 hrs.	1	Jerky breathing. Prompt recovery to normal

* The four animals used in this experiment varied no more than 5 gm. in weight.

chest was opened immediately after the animal had stopped breathing, the following conditions were found, although there was not the great regularity present in the same sort of examination made after a true anaphylactic death: The heart beating in block with both auricles and the right ventricle markedly distended; after death, clots in the ventricles; the lungs regularly as described as characteristic for heterophilic shock rather than that caused by ox serum,[5] except for the absence of a marked edema.

[5] Hyde, R. R., *Am. J. Hyg.*, 1927, vii, 1.

TABLE II.

Toxicity of Serum Following Treatment with Antisera.

Case No.	Interval after treatment	Serum sickness	Interval after serum sickness	Age of serum	Guinea pig reaction
13	5 days	None		6 days	None
	10 days	None		12 days	None
57	1 day	None		24 days	None
	21 days	None		9 days	Weakness. Light clonic seizures. Death in 20–30 min.
	29 days	None		7 days	Very jerky breathing and weakness for 30 min. Recovery
11	43 days	None		6 days	Jerky breathing. Weakness. Recovery
10	49 days	None		6 days	None. (1.5 cc. dosage)
34	2 yrs.	None		5 days	Weakness. Recovery
2, I	1 day	Immediate	Few hrs.	5 days	Death in 20 min.
14, I	47 days	Immediate	46 days	3 days	Death in 30 min.
II	67 days	Immediate	1 day	37 days	None
56, I	Before treatment			9 days	None
II	2 days	Immediate	1 day	8 days	None
8	6 days (also 1 yr. earlier)	Severe	Few hrs.	19 days	Prostration. Death in less than 2 hrs.
9, II	4 days	Local and general	Few hrs.	7 days	None
9, III	18 days	As in I	13 days	8 days	Convulsions. Death in 20 min.
				45 days	None. (Fatal anaphylaxis with 0.5 cc. transfer)

TABLE II—*Continued.*

Case No.	Interval after treatment	Serum sickness	Interval after serum sickness	Age of serum	Guinea pig reaction
7	12 days	Severe	5 days	15 days	None
2, III	2 days after last	Severe	6 days	8 days	Death in 4 min. (1 cc. inactivated serum caused only slight symptoms) 0.5 cc. dose: death in 4 min.
IV	13 days after last	Severe	20 days	8 days	Death in 4 min.
				45 days	Death in 15 min.
12	40 days	Severe	36 days	11 days	None
1	49 days	Severe	43 days	4 days	Doubtful
33	64 days	Light	60 days	5 days	None
56, III	5 days	Moderate	Few hrs. after onset	5 days	None
IV	8 days		3 days	11 days	Jerky breathing. Recovery
V	11 days		6 days	8 days	As in IV
VI	14 days		9 days	5 days	Jerky breathing for 20 min. Recovery
VII	19 days		14 days	10 days	None
VIII	30 days		25 days	6 days	None
55, I	Before treatment			14 days	None
II	4 days	None		10 days	None
III	7 days	None		7 days	Jerky breathing. Definite continued clonic seizures. Death in 30 min.

TABLE II—*Concluded.*

Case No.	Interval after treatment	Serum sickness	Interval after serum sickness	Age of serum	Guinea pig reaction
IV	11 days	None		8 days	Slight weakness only. (Experiment repeated with same results)
V	14 days	Very mild	1 day	5 days	Clonic seizures in 3 min. Death in 5 min. Froth at mouth and nostrils
VI	18 days		5 days	7 days	None
58, I	Before treatment			10 days	None
II	3 days	None		7 days	Jerky breathing. Recovery
III	7 days	None		3 days	Clonic seizures. Recovery
IV	10 days	Severe 3–4 days	1 day after onset	5 days	None
V	14 days		5 days	8 days	None

II. Effect of Treatment with Antisera upon Primary Toxicity of the Serum.

The findings as to toxicity of post treatment sera are given in Table II, where the cases have been grouped according to their response to the antiserum. If only a fatal reaction in the guinea pig is considered as a criterion of toxicity in the serum, it may be noted that this phenomenon occurred in only one (Case 57) of the five instances in which a history of serum sickness was lacking, and not at all in any of the 14 cases without history of treatment.

Of the 19 cases in which horse serum had been administered, 11 furnished serum definitely toxic for the guinea pig in a 1 cc. dose, but only in those cases where a definite serum sickness occurred (except

in the case noted above) and where the interval between this and the time of bleeding lay between the 5th and the 20th day. The exception is in one instance of an accelerated serum sickness (Case 2, I) where the sheep cell agglutinin titer at this time suggests the explanation of an already existing unusually high content of heterophilic antibody. It must also be noted that two factors may have brought the positive findings for toxicity to a lower number than would have obtained if these could have been controlled, namely, the interval after treatment at which the bleedings were made, and the use only of guinea pigs known to be non-complement deficient.[6]

In these examples then, 1 cc. of serum in many cases of serum sickness where the antibody response is general, would seem to contain an amount of the toxic factor comparable to that found by Redfern[7] in heterophilic immune rabbit serum, where the minimum lethal dose was about 1 cc. for slightly larger animals than those used in our experiments.

The manifestations presented by the animals after injections of toxic sera were, generally, of the type described by that author; that is, the symptoms could not be differentiated from those characteristic of typical anaphylaxis, except that occasionally an animal collapsed before it could be removed from the operating board, and showed no other evidence of life beyond a few terminal gasps; and in certain other instances, the animal would breathe so jerkily as to move the whole body for a few minutes and then later suddenly fall to one side without the usual manifestation of definite clonic seizures, make running motions, become quiet, lie thus for some moments, and finally cease breathing with a few gasps. Bloody froth from the mouth and nose occurred, but not regularly.

The lungs were uniformly hemorrhagic; sometimes punctate, sometimes splotched with diffuse or solidly dark red areas. In section, a corresponding appearance was found; occasionally, the whole center of the lobe would be dark red to within a millimetre of the pleura. The heart was always found engorged; and, if it had stopped beating, the auricles and right ventricle were seen to be markedly distended.

[6] Hyde, R. R., *Am. J. Hyg.*, 1927, vii, 619.

[7] Redfern, W. W., *Am. J. Hyg.*, 1926, vi, 278.

The primary toxicity of the serum was capable of modification by three factors: heating to 56°C. for 30 minutes, absorption by guinea pig kidney pulp, and ageing. The last factor was operative in one specimen after 45 days, when lethal toxicity had disappeared; but in another, this had persisted apparently unchanged after the same interval.

When the surviving animals, including those injected with inactivated or absorbed serum, were reinjected 48 hours later with horse serum (1 cc.) typical violent anaphylaxis occurred in 21 cases out of 33; but 5 of the guinea pigs not having shown primary toxicity, and 11 having shown the latter did not exhibit anaphylactic symptoms. This discrepancy in antibody content is particularly evident in Case 55 (Table II), in which none of the usual antibodies to be found following serum sickness could be demonstrated at any time. This is then apparently an example of individual variation in antibody response.

The interval between the onset of serum sickness and the time at which the bleeding was made was shown to be an important factor in the demonstration of toxicity, as it is for regular antibody production,[3] as shown in Case 1, where the toxicity was marked in serum taken 21 days after the onset of the rash, but had disappeared by the 35th day.

III. Effect of Absorption of Toxic Serum with Rabbit and Guinea Pig Kidney Suspensions.

In an effort to answer the question as to whether or not the source of the observed toxicity lay in a heterophilic antigen and immune serum reaction (of the Forssman type), differential absorptions of the sera were made as follows:

Rabbit and guinea pig kidney suspensions were made by cutting an organ into small pieces, rinsing thoroughly with saline, and pulping in a mortar with equal parts of saline. A volume of two proven fatal doses of the serum was treated with 2 capillary drops of the guinea pig kidney suspension, and a like preparation was made with the rabbit kidney for control. These were kept 15 minutes in the water bath at 37°C. and 45 minutes in the ice box. These preparations, after centrifuging out the kidney pulp, were then injected into two animals

of the same size. The whole experiment was carried out on the same day. Typical results are shown in Table III.

In four of seven sera studied for this phenomenon, when a double fatal dose of the toxic sera was absorbed differentially by rabbit and guinea pig kidney emulsions a definite to marked difference was

TABLE III.

Effect of Absorption upon Toxicity.

Case No.	Age of serum	Guinea pig reaction following injection of serum absorbed by:	
		Rabbit kidney emulsion	Guinea pig kidney emulsion
	days		
1	5*	Slight jerky breathing	None
	6	Weakness for an hour	Slightly difficult breathing
2, II	3*	Immediate death	None
	8†	Convulsions; death in 8 min.	Convulsions; recovery at 9 min.; convulsions again at 60 min. Death at 103 min. (Exhaustion?)
3	8‡	Violent seizures; death in 5 min.	None
5§	7	Death in 7 min.	Slight clonic seizures only
7§	4	Convulsions; death in 15 min.	Convulsions; recovery
9¶	3	Convulsions; death in 2 min.	Weakness after 30 min.
	8	Prostration; death in 40 min.	Slight jerky breathing

* Single lethal dose of serum absorbed.

† Blood taken 6 days after serum sickness.

‡ Single lethal dose, 1.5 cc. (in other cases 1 cc.).

§ Blood taken on day of serum sickness.

¶ Blood taken 5 days after first serum sickness (on 1st day of second sickness).

shown, the rabbit kidney-absorbed serum in every case manifesting the greater toxicity. These results favor the view that for these human immune sera the guinea pig kidney contained an increase of a specific reacting antigenic substance (heterophilic antigen) over that found in the rabbit kidney. But there is evidence, in the difficulty found in adjusting proper dosage for the absorption tests, that there

may be also a common absorbing substance analagous to the group reaction differences found in other immune phenomenon; and there is, too, the alternative possibility that this common absorbing factor may be due entirely to the common purely physical property of absorption of one colloid by another.

SUMMARY.

From the foregoing study there are indications:

1. Of toxicity in all fresh human serum for the guinea pig; this toxicity tends to disappear after 48 hours after bleeding, and its manifestations are strikingly similar to those of the heterophilic antibody in immune rabbit serum.

2. Of an increased toxicity in the serum of antisera-treated human cases; this is usually coexistant with the production of other antibodies; it tends likewise to disappear in time after treatment; differential absorption experiments indicate that its character is heterophilic, and its manifestations differ from those of anaphylaxis in that certain circulatory effects—hemorrhage and increased edema in the lungs and distension of the right heart—are added to the usual findings in true anaphylactic deaths.

OBSERVATIONS ON THE OXIDATION-REDUCTION PROPERTIES OF STERILE BACTERIOLOGICAL MEDIA.

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INTRODUCTION.

Avery and Neill (1) have shown that the pneumococcus cell contains active oxidation-reduction systems. It is unnecessary to present again their conception of the nature of these systems, or the different oxidation-reduction processes exhibited by them. However, it is important to point out that the bacterial cell must not be considered as a mere catalyst in these reactions. On the contrary, when the cells-substratum system is isolated, there must exist a quantitative relationship between reduction (or oxidation) of the substratum, and oxidation (or reduction) of the cells. If we keep for instance a mixture of pneumococcus cells and methylene blue under anaerobic conditions, a reduction of the dye will be observed, the cells constituting the reducing agent, and some at least of the cell constituents becoming oxidized in the process. It is to be expected that the fundamental properties of the cells are affected by such oxidation.

As will appear from the following pages, plain broth itself contains an active oxidation-reduction system. The condition of this system probably influences the condition of the cell.

In the course of studies on the initiation of growth and on the death rate of certain facultative anaerobes, and the toxic action exerted on them by certain dyes and metal salts, it was recognized that these phenomena are often controlled by the conditions of oxidation-reduction prevailing in the medium. Some knowledge of the oxidation-reduction properties of sterile bacteriological media is necessary for an understanding of these biological happenings.

HISTORICAL.

That sterile bacteriological media are not inert from the point of view of oxidation-reduction processes is an old observation. The oxidation of sterile broth by atmospheric oxygen, especially under the influence of light, has attracted attention since the beginnings of bacteriology (2). As early as 1887, Spina (3) noted the reduction of methylene blue by sterile nutrient gelatin. In 1896, Smith (4) reported that, in the closed arm of fermentation tubes, methylene blue, indigo sulfonate and litmus could be reduced by sterile media. Methylene blue was most rapidly reduced, indigo sulfonate less quickly and litmus only when sugar had been added to the medium. The addition of glucose to the broth and the application of heat always accelerated the reduction. Aqueous solutions of glucose and peptone were found to be inert. The reduction of methylene blue by sterile media was also studied by Gates and Olitsky (5); they used it as a test for the removal of oxygen from the medium, following the addition of sterile tissue.

While there are found in the literature many studies of the speed of reduction of methylene blue by bacteriological systems, an analysis of the reduction phenomena in terms of "intensity" and "capacity" does not seem to have been made. The importance of these factors was not properly emphasized until the work of W. M. Clark. It may be useful to present briefly points he has outlined that bear directly upon the present work.

Oxidation is not necessarily a reaction characterized by the addition of oxygen to a molecule; the term applies also to loss of hydrogen; more essentially, oxidation consists in a loss of electrons, and reduction in a gain of electrons. This implies that oxidation of one substance necessarily involves the reduction of an equivalent amount of another, the former giving electrons to the latter. The tendency of a system to reduce another may be measured by its tendency to give up electrons to it. This tendency expresses itself in electrometric potentials and is used as a means to measure the factor intensity in processes of oxidation-reduction. Of two systems, the one which has the greater tendency to take up electrons has an electropotential positive to that of the other. Instead of measuring the potential of two reversible oxidation-reduction systems one against the other, it is more convenient to measure them separately against a standard (the standard hydrogen electrode). We therefore speak loosely of the potential of a system (as measured against the hydrogen electrode) as a numerical index of its oxidation-reduction intensity. In the case of a definite reversible system (such as methylene blue-methylene white), the potential of this system is a function of the ratio, reductant to oxidant $\left(\frac{\text{methylene white}}{\text{methylene blue}}\right)$, of the pH of the system, of the temperature, etc. Such factors should be defined in describing the system.

It is apparent that there are two means of measuring the intensity of oxidation-reduction of a system. One is a measurement of the electrode potential, the other a determination of the percentage reduction of certain dyes which are themselves reversible systems of oxidation-reduction. The reduction intensity is usually

expressed numerically in terms of potentials against the hydrogen electrode. Clark has also introduced the symbol rH which stands for the negative logarithm of the hypothetical hydrogen pressure in equilibrium with the system in question. Both terms may be used to define the position of the indicators on the scale. The E_0' of an indicator is the "potential" of a mixture of equimolecular amounts of oxidant and reductant of that indicator, at a definite pH; the rH also corresponds to a ratio $\left(\frac{\text{reductant}}{\text{oxidant}}\right) = 1$, but is independent of the pH.

The higher its E_0' (or its rH), the more rapidly an indicator is reduced; on the other hand, the reduced forms of the dyes having the smaller E_0' (or rH) are the more readily oxidized. Any system is an oxidizing agent in relation to another with a more negative "potential," and a reducing agent for the more positive systems.

It must be understood that the potential (E_0' or rH) is only a measure of *intensity*, but not of total *capacity* to oxidize or reduce. We have here the same relation as between the intensity factor (pH) and capacity factor (total acidity) in the description of the acid-base systems. Two systems of the same oxidation-reduction potential (intensity factor) may differ greatly in the amount of substance they are capable of oxidizing or reducing (capacity factor).

The experimental observations which follow are not presented as a quantitative study of the oxidation-reduction properties of plain broth, but only as an introduction to the problems of growth and dye toxicity which form the subject of subsequent papers.

EXPERIMENTAL.

Methods.

Of the two methods of determination of the oxidation-reduction potential of a system, the electrometric one is probably preferable as it gives results without the necessity of introducing a foreign factor (dye indicator) into the medium. The indicator being itself a system of oxidation-reduction acts to "poise"[1] the medium at its own special potential. We may even conceive of a medium so poorly "poised" that it will take automatically the potential of the indicator. Furthermore, the colorimetric reading of the percentage reduction of the dye, by which the potential is determined, is only approximative.

However, the colorimetric method has been adopted in preference to the electrometric one for the following reasons: (*a*) media containing the indicators in oxidized and reduced forms have been used in certain biological studies which will

[1] A solution is said to be "poised" when it tends to resist change in oxidation-reduction potential on addition of an oxidizing or reducing system. This effect is comparable to the buffer action found in the acid-base system.

be reported later; (*b*) the indicator method allows some measure of the amount of reducing substance present in the medium.

In Table I is given a list of the indicators used in these studies. Their potentials have been measured by Clark and his coworkers (7) except in the case of Janus green, neutral red and phenosafranine. The potentials of these three have been measured by Rapkine and Würmser (8) and the values are only approximative. The Janus green molecule corresponds to two systems of oxidation-reduction; the green form changes to pink at a potential of $E_0' = -0.02$ volt (pH = 7.4), rH = 13; the pink form changes to the colorless at $E_0' = 0.26$ volt (rH = 5). Only the latter system pink ⇄ colorless is reversible; for the pink form does not turn green even if oxygen is bubbled through it. Table I also contains two dyes (litmus and malachite green) for which there are no measurements available in the literature, although they constitute reversible systems of oxidation-reduction. Considering the difficulty with which they are reduced by bacterial cultures, it may be suspected that they are negative to methylene blue and to some, at least, of the indigoes.

TABLE I.

List of Dyes Used.*

Name of oxidant	E_0' (pH 7.4) (in volts)	rH
2-Chloroindophenol (o-chlorophenol indophenol)	+0.233	21.8
Indophenol (phenol indophenol)	0.228	21.6
2-Methyl indophenol (o-cresol indophenol)	0.195	20.5
1-Naphthol-2-sulfonate indophenol	0.123	18.1
Methylene blue	0.011	14.4
Janus green (green⟶pink)	−0.02 approx.	13.0
K_4 indigo tetrasulfonate	−0.046	12.5
K_3 indigo trisulfonate	−0.081	11.3
K_2 indigo disulfonate	−0.125	9.9
K indigo monosulfonate	−0.182	7.5
Janus green (pink⇄colorless)	−0.26 approx.	5.0
Neutral red	−0.31 approx.	3.7
Phenosafranine	−0.525 approx.	−3.5
Litmus	?	?
Malachite green	?	?

* The indophenols, the methylene blue, and 3 of the indigoes were obtained from La Motte Chemical Company. Indigo trisulfonate was obtained through the courtesy of Dr. B. Cohen of the Hygienic Laboratory, Washington D. C. The other indicators were dyes used in the laboratory for staining and cytological work.

The concentrations of the dyes have been measured in terms of molar concentrations. The solutions were prepared in distilled water shortly before use. They were all autoclaved, except the four indophenols which are heat-labile. The solutions of indophenols were prepared under aseptic conditions and proved to be sterile.

Most of the dyes were rapidly decomposed in plain broth, especially under aerobic condition. This factor introduced an element of error, due to a change in the relative concentrations of the different dyes and the effect of decomposition products. Most unstable were the indophenols, especially phenol indophenol and 2 methyl indophenol; this unstability, added to the weak tinctorial power of these two dyes, was responsible for discontinuance of their use. The color of the

TABLE II.

Time Required for the Reduction of Indicators at Concentrations of 0.0002 M by "Boiled" Plain Broth (pH 7.8) Kept under Vaseline Seal.

2-6 Chloroindophenol	2 hours	2 hours or less	Indigo monosulfonate	Not reduced after a month
Phenol indophenol	2 "		Janus green (pink⇌colorless)	
2-Methyl indophenol	2 "		Neutral red	
1-Naphthol-2-sulfonate indophenol	7 "		Phenosafranine	
Indigo tetrasulfonate	20 "		Litmus	
Methylene blue	52 "		Malachite green	
Janus green (green→pink)	72 "			
Indigo trisulfonate	110 "			
Indigo disulfonate*	288 "			

* The broth-indigo disulfonate mixture presented a very slight greenish tinge which did not disappear in the course of a month.

indophenols and indigoes rapidly faded in aerobic tubes of broth (in less than 12 hours). As the color could not be restored by the addition of potassium ferricyanide, it is apparent that this change in color was not due to reduction by the broth; it seems on the contrary that oxidation by air results in a breaking down of the molecule since the dye-broth mixtures kept under vaseline seal largely recovered their original color following addition of potassium ferricyanide. The sample of indigo monosulfonate partly precipitated out in plain broth, and the results obtained with such preparations may be open to question.

To prevent the access of air, the tubes were sealed with a 2 cm. layer of sterile vaseline.

Most of the experiments were carried out with a batch of meat infusion broth (pH 7.8) prepared 2 to 4 weeks before use. Fairchild's peptone (lot 280630) was used.

Reduction of Dyes by "Boiled" Broth.

As reported in the historical review, sterile plain broth is capable of reducing methylene blue when protected from the action of oxygen. The object of the first experiment was to establish whether other indicators of oxidation-reduction would be reduced, and if so, in what order.

Experiment 1.—Sterile plain broth (3 weeks old) was tubed in amounts of 5 cc. The tubes were boiled for half an hour to deaerate the broth, rapidly cooled down and enough of the dyes added to give a final concentration of 0.0002 M. The tubes were then sealed with vaseline and incubated at room temperature (25°C.). The time required for reduction in each instance is given in Table II.

A first conclusion to be drawn is that, under the conditions of the experiment, the reduction time of equimolecular concentrations of the different indicators decreased with the increase of the electrode potentials, thus confirming Voegtlin's conclusion that "the indicators if tested by biological methods arrange themselves in the same order as that obtained by means of the purely physical electrode measurements" (6). Especially remarkable is the fact that Janus green comes immediately after methylene blue, although its reduction (green → pink) is not a reversible process; this seems to offer some support to the theory of "apparent oxidation-reduction potentials" of irreversible systems, suggested by Conant (9).

Concerning the order of reduction of the different dyes, the only anomaly is that of indigo tetrasulfonate which was reduced in less time than methylene blue. This fact has been confirmed by duplicate experiments; its probable explanation is that a partial breaking down of indigo tetrasulfonate takes place in presence of the broth, a fact already discussed.

The reduction of indigo monosulfonate must have been only very slight; the partial precipitation of the dye prevented accurate reading. Neutral red, phenosafranine, malachite green and litmus were not reduced at all, and Janus green was not reduced from the pink to the colorless form. These facts indicate that the potentials of litmus and malachite green will be found on the negative side of indigo disulfonate.

Indigo disulfonate was only slowly reduced and it is even possible that its reduction was never complete as the broth-dye mixture

maintained a faint greenish tinge. It seems, therefore, that the plain "boiled" broth kept under vaseline seal slowly reaches a potential corresponding to a very advanced reduction of indigo disulfonate (at pH 7.8).

The fact that indigo disulfonate perhaps was not completely reduced may be an indication that, although the broth is capable of reaching more reducing potentials, it does not contain enough reducing sub-

TABLE III.

Time Required for the Reduction of Different Concentrations of Indigo Disulfonate by "Boiled" Plain Broth under Vaseline Seal.

Concentration of the dye	0.002 M	0.001 M	0.0005 M	0.0002 M	0.0001 M
Time at which the final stage of reduction was reached	16 days 50 per cent reduction	16 days	14 days	12 days	8 days
		All tubes reach the same very faint greenish tinge			

TABLE IV.

Reducing Capacity of "Boiled" Plain Broth for Dyes of Different E_0'.

Concentration of the dye	0.002 M	0.001 M	0.0005 M	0.0002 M
	per cent reduction			
2-6 Chloroindophenol	60	Complete reduction		
1-Naphthol-2-sulfonate indophenol	80	"	"	
Methylene blue	50	"	"	
Indigo tetrasulfonate	70	"	"	
Indigo disulfonate	70	Very faint greenish tinge		

stances to reduce a 0.0002 M concentration of the dye. Experiment 1 was therefore repeated, using different concentrations of indigo disulfonate (Table III).

It appears that the broth used in these experiments reaches a final potential corresponding to almost complete reduction of indigo disulfonate. However, with another lot of broth, prepared only 24 hours before the experiment, complete reduction of the dye was obtained in 3 days.

It is possible to conceive of a medium containing several systems of oxidation-reduction which have not reached a condition of equilibrium,

and which it is permissible to call "independent systems." If the broth used in these experiments contains several such "independent systems," it may be expected that they will not all have the same reduction potential; under such circumstances, the broth would reduce a larger amount of dyes with higher E_0', than of dyes with a lower E_0'.

To test this possibility, the reducing *capacity* of the broth for dyes of different E_0' was measured, concentrations of dyes varying from 0.002 M to 0.0002 M being used.

Experiment 2.—The tubes of broth-indicator mixtures (5 cc. per test-tube) were sealed with vaseline and incubated at room temperature. The final readings were made after 3 weeks (Table IV).

With the possible exception of indigo disulfonate (the case of which has already been discussed), the broth reduces completely all the dyes in concentrations of 0.001 M but only partially the 0.002 M concentrations. The differences between the different dyes may be accounted for by the partial breaking down of some of them in the presence of broth (see under Methods).

On the whole, the experiment seems to indicate either (*a*) that the broth contains only one active system of reducing substances (at least in appreciable amounts), or (*b*) that, if there are several "independent" systems, their rH is about the same.

The boiling of media has usually been resorted to as a means of driving out the oxygen in solution, and it has been considered that we were dealing here with a purely mechanical action. There is considerable biological and chemical evidence which suggests that this is not the case. The biological evidence will be presented in a subsequent paper.

The facts observed when the oxidized indicators are added to "boiled" broth will now be considered in some detail.

Experiment 3.—Test-tubes containing 5 cc. of plain broth (prepared 1 week before the experiment) were boiled for 1 hour (or autoclaved), and rapidly cooled down; enough dye was then added to give a final concentration of 0.00025 M.

The 2-6 chloroindophenol turned colorless immediately after addition to the "boiled" broth, but the original color could be restored by the addition of potassium ferricyanide; this shows that the dye had been reduced and not decomposed.

Another tube similarly treated, but without addition of potassium ferricyanide, was aerated by shaking, but the dye remained colorless. The tube was kept opened (with only a cotton plug to insure sterility) and it took more than 6 hours before the dye turned blue again.

The results were the same when phenol indophenol was used. With 1-naphthol-2-sulfonate indophenol, the reduction was only partial and the reoxidation more rapid. With methylene blue, the phenomenon was hardly noticeable, although there was for a few minutes, a partial reduction of the dye. Nothing was observed with the other indicators.

These results are hard to explain on the assumption that the only effect of boiling is a mechanical removal of oxygen. If it were so, the dye should be oxidized immediately after readmittance of oxygen (as provided by shaking). Three possibilities which may be suggested are, that boiling results in (1) the breaking down of some oxidizing substance in the broth, (2) the liberation of some reducing substance, (3) the reduction of some reversible system of oxidation-reduction; the third hypothesis would of course imply the oxidation of some other constituent of the broth.

The progressive reduction of dyes by "boiled" broth can be shown more rapidly by the following technique:

Experiment 4.—Tubes of broth containing 0.0002 M of the different dyes were immersed in a bath of boiling water. The indophenols could not be used in this experiment because they are not heat-stable. Under such conditions, the dyes were reduced in the following order: indigo tetrasulfonate, methylene blue, Janus green (green → pink), indigo trisulfonate, indigo disulfonate. The reduction of indigo disulfonate was only partial and took several hours, while the first three dyes were reduced after 1 to 2 hours boiling. The dyes negative to indigo disulfonate were not affected.

Reduction of Dyes by "Unboiled" Broth.

It has been shown that a progressive drift toward high reduction potentials can be observed in broth-dye mixtures sealed with vaseline. Even though the broth was boiled immediately before the experiment, it had time to dissolve some oxygen before being sealed. As all the dyes down to indigo disulfonate were maintained in the reduced condition under the vaseline seal, it may be supposed that the oxygen in solution had combined with some element of the broth. This fact is much more clearly evidenced when "unboiled," instead of "boiled" broth is used.

Experiment 5.—The same procedure described in Experiment 1 was used, except that half of the tubes of broth were not boiled, while the other half were boiled for 1 hour immediately before the experiment. The broth was 3 weeks old. The dyes were used in final concentrations of 0.001 M, 0.0005 M, 0.0002 M. The tubes, sealed with vaseline, were incubated at room temperature. In Table V are given the number of hours required for 30 per cent, 60 per cent and 90 per cent reduction of the indicators.

TABLE V.

Time (in Hours) Required for the Reduction of Dyes by "Boiled" and "Unboiled" Broth.

Dye	Concentration	"Unboiled" broth			"Boiled" broth		
		30 per cent, red	60 per cent, red	90 per cent, red	30 per cent, red	60 per cent, red	90 per cent, red
	M						
2-6 Chloroindophenol	0.001	—	—	—	—	3	7
	0.0005	5	20	144	—	3	7
	0.0002	3	15	30	—	—	3
1-Naphthol-2-sulfonate indophenol	0.001	—	72	144	7	20	72
	0.0005	7	20	94	—	7	20
	0.0002	3	20	72	—	3	7
Methylene blue	0.0005	94	144	288	29	48	192
	0.0002	48	72	144	3	7	72
Janus green	0.0002	168	240	292	—	20	72
Indigo tetrasulfonate	0.001	94	144	168	48	72	144
	0.0005	17	48	168	7	20	48
	0.0002	—	48	72	—	—	20
Indigo trisulfonate	0.0002	48	94	240	21	48	110
Indigo disulfonate	0.001	—	192	336	21	144	336
	0.0005	72	172	336	72	144	336
	0.0002	48	94	288	—	—	288

Numerals = time in hours
— = test not made

Several conclusions may be drawn from this experiment.

Whether or not oxygen is present in large amounts in the broth at the beginning of the experiment, the final reduction intensity reached under vaseline remains the same (at least as far as could be measured by the colorimetric method). The order of reduction is much more regular in "boiled" than in "unboiled" broth. The time of reduction is greater in "unboiled" than in "boiled" broth. The difference is

considerable with the dyes having a high E_0', but is almost insignificant in the case of the indigo disulfonate. The following considerations may account for this fact. The indophenols are rapidly reduced, but reoxidized as fast as reduced by the oxygen present in the broth. On the contrary, the process of reduction of the indigo disulfonate is extremely slow. While it is going on, the oxygen itself combines with some autoxidizable agent of the broth, so that only a negligible fraction of the reduced dye is reoxidized by free oxygen.

One fact of special interest is that the experiment demonstrates the presence in plain broth of some autoxidizable substance which combines with free oxygen. The pressure of oxygen corresponding to the amount dissolved in the broth has not been computed, but this pressure is certainly too high to be in equilibrium with the reduced dyes. That free oxygen has combined in some way is therefore not doubtful. This oxygen may perhaps combine with the reducing system responsible for the dye reduction. If this system is reversible, the presence of different concentrations of oxygen in the broth may affect the final potential, and the difference would be revealed by potentiometric measurements. On the other hand, the oxygen may combine with some substance other than that responsible for the reduction of the dye, and form a highly oxidized agent of the nature of a peroxide.

Is the Reduction of Dyes "Catalyzed" by Broth?

Although the reduction of methylene blue by plain broth has been used as a test for the "absence" of oxygen in anaerobic jars, the mechanism of this reduction has been little understood. The object of the following experiment was to test whether broth can act as a catalyst in the reduction of methylene blue in the presence of hydrogen.

Experiment 6.—Sterile broth containing 0.0002 M of the different dyes was transferred in 10 cc. amounts to test-tubes which were closed by a rubber stopper with glass connections to allow the bubbling of hydrogen gas (Fig. 1), all points of contact between glass and rubber being sealed with vaseline. The hydrogen was not completely oxygen-free as it had been washed only through potassium permanganate.

When hydrogen was bubbled through the broth-dye mixture (at a pressure of 2 lbs.), all the dyes with a rH positive to that of indigo trisulfonate were completely

reduced. Indigo trisulfonate and disulfonate were not reduced even after 10 hours treatment. The bubbling was discontinued at that time.

When palladinized asbestos was added to the broth-dye mixture, and hydrogen bubbled under the same conditions, all the dyes were rapidly reduced.

This experiment shows that the broth is not capable of catalyzing the reduction of dyes by hydrogen.

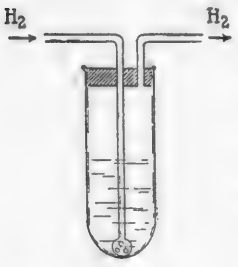

FIG. 1

It may be of interest to mention that there is in broth a substance which, under certain conditions, can increase the velocity of reduction of methylene blue by cysteine.

Experiment 7.—5 cc. portions of a $\frac{1}{100,000}$ (by weight) solution of methylene blue in phosphate buffer (pH 7.5) were transferred to test-tubes, and enough cysteine hydrochloride (neutralized with NaOH) added to bring its final concentration to 0.05, 0.03, 0.02, and 0.01 per cent respectively. Some of the tubes received also 0.1 cc. of broth (recently prepared), some 0.1 cc. yeast extract and others were kept as controls. The tubes were sealed with vaseline and incubated at 37°C. The times of reduction of the methylene blue in the different tubes are given in Table VI.

TABLE VI.

Time (in Minutes) Required for the Reduction of Methylene Blue (Concentration 1/100,000) by Cysteine.

	Concentration of cysteine				
	0.05 per cent	0.03 per cent	0.02 per cent	0.01 per cent	0
Phosphate buffer (pH 7.5)	60	120	150	Reduction only partial	No reduction
Phosphate buffer (pH 7.5) + 2 per cent broth	12	30	40	"	"
Phosphate buffer (pH 7.5) + 2 per cent yeast extract.....	12	25	40	"	"

This experiment brings out the interesting fact that the reduction of methylene blue by cysteine is activated by small amounts of broth and yeast extract. To study now the mechanism of this activation would lead us too far astray.

Is the Reducing Power of the Broth Due to a Reversible System of Oxidation-Reduction?

It is a matter of common observation by those who use broth-methylene blue mixtures as indicators of removal of oxygen from anaerobic jars, that the same tube of methylene blue broth may be used a number of times, the methylene blue being alternately oxidized when brought in contact with the air and reduced again in the anaerobic jar. If there exists an equilibrium between the methylene blue and the reducing substances of the broth, this fact is an indication that there exists in the broth a reversible system of oxidation-reduction. But it is also possible that each reduction of the methylene blue corresponds to the irreversible oxidation of an equivalent amount of reducing substances in the broth and that the process would stop after a certain number of reductions.

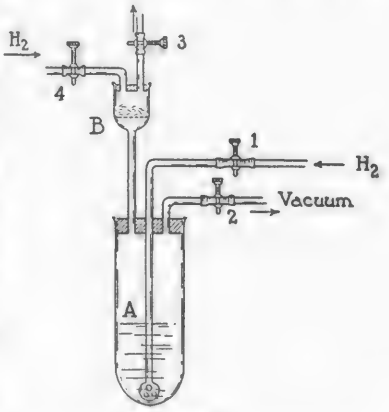

FIG. 2

The following experiment bears on this point.

Plain broth, kept under vaseline seal, reduces all the dyes with an E_0' greater than the potential of indigo monosulfonate, but does not affect the more negative dyes. If the oxidation-reduction system of the broth is reversible, it should be able to oxidize the reduced forms of these dyes. This was shown to be true by the use of the apparatus presented in Fig. 2.

Experiment 8.—*A* is a test-tube containing the broth to be studied, *B* a Jena glass filter containing palladinized asbestos and the dye to be reduced. The glass rubber connections were sealed with vaseline; the hydrogen was bubbled at a pressure of 2 lbs., this hydrogen had been washed only through potassium permanganate.

The procedure was as follows: The whole apparatus was washed through with hydrogen, then, the stop-cocks 2 and 4 being closed, a current of hydrogen was

passed for 1 hour through the broth which had been boiled immediately before; this stream of hydrogen served to deaerate the broth and reduce the dye in *B*.

After the dye had been completely reduced, the stop-cocks 2 and 3 were closed, and 2 and 4 opened. The reduced dye was thus forced down into the broth through the glass filter, enough dye being used to give a final concentration of 0.0002 M.

The method could not be used for methylene blue owing to the very low solubility of the leuco base and also to the absorption by the glass filter.

The following indicators were used: 2–6 chloroindophenol, indigo tetrasulfonate, neutral red, phenosafranine. The results may be summarized as follows: Reduced neutral red and phenosafranine were oxidized as soon as they came in contact with the deaerated broth. The other dyes remained reduced.

DISCUSSION.

As mentioned in the introduction, the object of this survey was not a systematic study of the oxidation-reduction system of plain broth, but only a *description* of the behavior of broth in the presence of dyes, under the conditions used in other biological studies. However, it may be of interest to analyze the results as a means of developing a working hypothesis for a systematic and accurate investigation of the problem.

The term "reducing power" of the broth has been resolved into its three components: the intensity factor (reduction potential), the capacity factor (amount of dye reduced) and the time factor.

When the system plain broth is protected from oxygen, it reaches an ultimate reduction potential corresponding to reduced indigo disulfonate; dyes with a more negative E_0' are not reduced. Equimolecular quantities of the dyes are reduced, indicating that there is either a single system active in the broth, or that if several "independent" systems are present, they all have the same reduction potential. The indicators are reduced progressively in the order of the electromotive series, with the exception of indigo tetrasulfonate which is reduced faster than would be expected; this exception is probably accounted for by a partial breaking down of the dye in the broth.

As regards the nature of the reducing substance present in the broth, it has usually been considered to be a reducing sugar (10); the following considerations throw some doubt on this assumption: The lot of broth used in this work could reduce an amount of dye

corresponding to a concentration of about 0.0017 M. Other determinations show that this reducing capacity is unusually low and that other lots of broth can reduce at least 4 times as much. If this reduction were due to a sugar, the broth should contain an equivalent amount of this reducing sugar, *i.e.*, 0.025 to 0.1 per cent in terms of glucose. In view of the great improvement of growth (with such an organism as Pneumococcus) obtained by the addition of 0.03 per cent glucose to plain broth, it seems unlikely that such broth already contains 0.025 to 0.1 per cent glucose, although the possibility remains that broth contains a sugar very different from glucose in its biological properties.

It has been seen that a number of dyes are reduced by plain broth when the mixture is kept under a vaseline seal. As already mentioned, it is hardly possible that the dyes exist in the reduced form in presence of the oxygen dissolved in the broth, and, consequently, it is concluded that this oxygen combines with some autoxidizable substance of the broth. If this reaction goes on under vaseline seal, it must also go on under aerobic conditions.

This oxidation by molecular oxygen seems to be a slow process. It is possible, but not certain, that oxygen combines with the reduced system responsible for the reduction of the dye. If this system gives rise to a potential determined by the ratio $\frac{\text{reductant}}{\text{oxidant}}$, the final potential should be affected by the amount of oxygen which has combined with the system, in other words, by the age and aeration of the broth; one would then expect the potential of the completely reduced broth to be more negative than indicated by the results reported here. In fact, some experiments recently carried out with broth freshly prepared gave a *complete* and rapid reduction of indigo disulfonate.

On the other hand, the problem is complicated by another fact. When broth or nutrient agar are exposed to the action of air, at least in the presence of light, an oxidation takes place which seems to result in the formation of a substance having the characteristics of a peroxide. This factor should of course be taken into account. It is even possible that, as a result of oxidation, the same substance may give rise to the oxidized form of a reversible oxidation-reduction system and, at the same time, to a peroxide-like compound. Thurlow (11) has shown

for instance that, in the course of the autoxidation of glutathione by air, a peroxide is formed.

Finally, it may be proper to point out a fact of possible significance for anaerobic technique. It has been assumed that reduction of methylene blue in a medium or in an anaerobic jar corresponds to *complete* removal of oxygen (5). As a matter of fact, conditions may be obtained under which the broth is reduced enough to maintain methylene blue in the reduced condition, but not enough to reduce other dyes. In fact, Pasteur, in his work on anaerobiosis, used the reduction of indigo carmine as an index of anaerobic conditions. It is interesting to note that his standard was therefore much more exacting than ours, and it might be useful to come back to it in the cultivation of certain strict anaerobes.

SUMMARY.

Sterile plain broth contains an active oxidation-reduction system, the characteristics of which have been analyzed.

1. Intensity factor: Under vaseline seal, the lot of broth used in these experiments reaches a reduction potential corresponding to reduced indigo disulfonate (rH = 10). All the indicators with a more positive E_0' are reduced, the others are not affected.

It seems probable that fresh broth, which has not undergone oxidation by molecular oxygen, would give a higher reduction potential.

2. Capacity factor: The maximum amounts of different indicators that can be reduced correspond to equimolecular concentrations. This seems to indicate either (*a*) that the broth does not contain several "independent" reducing systems (at least in appreciable amounts), or (*b*) that these hypothetical "independent" systems all have about the same reduction potential.

3. Time factor: The different indicators of oxidation-reduction potentials are reduced in the order of the electromotive series.

4. Nature of the system: The system seems to be reversible (this not excluding the possibility of irreversible autoxidations) and does not appear to be of the nature of a sugar.

The relation of these phenomena to the cultivation of different species of bacteria will be reported later.

Addendum.—While this article was in course of publication, there appeared a paper by Coulter (12). By the electrometric method, he showed that if broth (pH 7.6) is deaerated by a current of oxygen-free nitrogen, this broth passes progressively from an initial potential between +0.250 and +0.150 volt, to a potential between −0.050 and −0.060 volt. "This appears to be a limiting value for the majority of lots of bouillon examined, and may be regarded as a characteristic potential for the system." If the flow of nitrogen is discontinued before the broth has reached the ultimate reduction potential, and the vessel sealed against the entrance of oxygen, "the potential continues to fall slowly and after 5 days or longer attains the limiting value above noted of − 0.060 to −0.050 volt."

Our own experiments confirm this drift toward a region of greater reducing intensity. However, the final potentials obtained by Coulter were appreciably positive to the ones reported in our experiments (the difference being in the neighbourhood of 0.100 volt). It is difficult to interpret the significance of these differences as long as comparative measurements by the electrometric and colorimetric methods have not been made. It is sufficient to recall here that, while electrometric measurements give to glutathione a reduction potential corresponding to a voltage of −0.063 volt, this substance can reduce indigo carmine, the E_0' of which is only −0.125 volt. For an explanation of this anomaly, see Kendall and Loewen (13).

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